

Pensioners mean problems

Modern governments have in common an obsession with getting pensioners off the backs of those still at work. They are in danger of ignoring the link with employment and of forgetting equity.

How, in the long run, can a modern state hope to deal equitably with its pensioners, especially if a substantial fraction of its putatively working population is forced into premature pensionhood by unemployment? And what if the social burden of the unemployment rolls will in any case be made permanent by developments in technology, especially by the prospect that the automation of production processes, after three decades of enthusiastic talk, is about to become a reality? These questions have been in the air for the past decade. They have become more urgent as the economic recession has been prolonged, and as some people have begun to suspect that economic recovery may, on this occasion, be marked by continuing stagnation of employment and thus by an unpalatable choice — either higher taxes or a widening of the gulf between those at work and those without jobs (pensioners included).

The question is far from being academic. For the past four years, a British government elected originally on the promise that it would reduce taxes across the board, and reinvigorate the economy by making it competitive, has seen social services consume a growing proportion of its budget and unemployment remain obdurately high (more than three million) in spite of flickering signs that economic activity is picking up. Demography offers no relief. The number of voluntary pensioners is certain to increase, the number of involuntary pensioners may remain large. Mrs Margaret Thatcher, the Prime Minister, has taken to pointing to the simple arithmetic of this set of circumstances — that consumption by pensioners can be financed only at the expense of those still at work, either by means of taxes or by that part of people's savings that does not find its way into capital investment. Mr Norman Fowler, Secretary of State for Health and Social Security, has gone further, putting himself at the head of a commission to look into the way in which state pensions in the United Kingdom should in future be financed. There are several interlocking issues, some practical and urgent (such as making sure that occupational pensions schemes do not inhibit mobility), some more distant but even more important. The success of Mr Fowler's inquiry will, however, depend crucially on the assumptions on which it is founded.

The first principle should be equitability. In most industrialized societies, people are compelled by law to contribute to pensions schemes separately from general taxation, presumably on the grounds that this simulates what happens when people contribute to private pensions schemes. Present consumption is forgone, postponed until after retirement. Governments are usually pleased with this arrangement, which is good for what is called their cash flow, but are less happy with the implied commitment to future pensioners. In some places, the United States for example, contributions are formally earmarked for the eventual purpose of paying pensions, but then promptly used to reduce the government's general need to borrow. Moreover, the existence of an earmarked fund does not absolve the government from the need to step in to top it up if it should seem deficient, so that the end effect is the same as in the British system. People pay an extra pension tax while they are at work and have the illusion that this "entitles" them to a pension but in retirement are paid as pension only what the government of the day decides it can afford.

In Britain, it was held to be a famous victory for the House of Commons that, some years ago, two private members were able to

amend the finance bill so as to link state pensions with the cost of living, but that is plainly unfair to pensioners if general prosperity is increasing. Worse still, however, is the general assumption by those who manage the government's business that pensions are an unmitigated drain on national resources, morally indistinguishable from a kind of poor relief. That might be so if it were not for the promise during pensioners' working lives that pensions would be payable. If Britain's Norman Fowler is looking for an equitable escape from present pensions problems, he might think of working towards a restriction of the numbers entitled to some kind of state pension, an offsetting reduction of what people pay — and then waiting until existing promises have been required before reaping the financial benefits.

Demography suggests another fruitful avenue for exploration. The post-war baby boom of the 1950s implies that the numbers of pensioners will be growing rapidly in the early decades of the next century, both in absolute numbers and as a fraction of the population in industrialized countries. Yet in most places, governments have not seriously tackled the problem of how best to encourage policies of flexibility on retirement. Throughout Western Europe, rigid definitions of retirement age still apply, taking no account of either increased longevity or changed social patterns, especially the growth of the female working population. (Japan and West Germany are two exceptions.) But the financial benefits to governments of flexibility are potentially huge — potential pensioners may remain productive. How great the benefits may soon become apparent in the United States, where discrimination on the grounds of old age is now illegal.

But will not a public policy that allows people to remain at work when they might retire merely exacerbate the problem of unemployment, itself almost intractable? This question begs that of whether technological developments now in prospect indeed entail the awesome consequences commonly attributed to them, factories run by robots and offices staffed by computing machinery, and with no jobs for the less skilled majority of any nation. Curiously enough, even those plotting these great technological upheavals are often among the first to beat their breasts and proclaim that they are awed by the enormity of what they are about. The truth, however, is that the present upheaval of technology in industrialized communities is a sign of yet another redistribution of labour in the sense in which Adam Smith intended the term. Some years hence, it will be found that some countries have entirely changed the pattern of their production. Some will have abandoned steel, or shipbuilding, for computer manufacture. Others will have stayed in the traditional industries. All will need agriculture, although policies of self-sufficiency will not be nearly justifiable as in the past. There is nothing new about this process (witness the 1870s). The outcome, invariably, is not merely a redistribution of labour between countries but a repartitioning of wealth as well. What matters most, to would-be pensioners, is that the economies on which they will ultimately depend should be among the stronger, which in turn argues for a transfer from public to private saving and for spending a greater proportion of private savings on capital investment. But how can a minister of pensions hope to tackle such important strategic questions, more properly the preserve of chancellors of the exchequer? Mr Fowler, in short, has taken on a gigantic task. Is he, by any chance, ambitious? □



What rights for animals?

The claim that animals have rights is not so much false as nonsensical and irrelevant.

ROBERT NOZICK, the Harvard University philosopher, wrote last week in the *New York Times* that animal rights is a subject that seems to attract cranks. The distinguishing feature of a crank, he was quick to point out, is not merely preoccupation with a single issue; the solving of the world's problems, after all, may well require a division of labour. What really defines a crank, Nozick said, is that he has no sense of proportion. If some of the correspondence provoked by a recent leading article on animal rights (*Nature* 13 October, p.562) is any guide, Nozick might have added to that definition a lack of a sense of humour. The more thoughtful correspondents (10 November, p.110; 27 October p.758), however, have raised some serious points that deserve a more serious answer. Is it permissible for humans to violate an animal's "right" to life only in those cases where strong moral arguments can be summoned to defend the action? How can animals be denied rights that severely retarded humans enjoy? What moral principles guide us in resolving the inevitably conflicting interests of humans and lower animals?

These are the questions that preoccupy philosophers and legitimate animal rightists. They are also totally unnecessary if one rejects from the outset — for the absurdity that it is — the notion that animals have "rights". Sarcasm perhaps failed to make that point; perhaps some commonsense arguments can. Simply, an animal is not a human being, nor an infant human being, nor a mentally retarded human being. An animal does not share human values, cannot grow up to be a being that shares human values, nor is a handicapped version of such a being. We need not even consider whether a healthy ape is a more free and feeling agent than a severely retarded human to recognize the uniquely corrupting effect on human values that comes of abridging the latter's rights. The real reason to defend the rights of severely handicapped humans is not that life is sacred in the abstract or that a being's ability to feel confers upon it an automatic right to exist; rather, it is the moral danger of allowing humans to pass arbitrary judgement on the rights of other humans. With good reason, we fear that society is unable to draw clear lines; crudely, once we admit the principle that select groups of humans can have their rights curtailed, it will not be long before someone will begin selecting on the basis of race, religion or political persuasion.

The same argument simply does not apply to animals. Even the most ardent advocate of animal rights acknowledges, explicitly or otherwise, a hierarchy of species. The ardent animal rightist does not bemoan the millions of bacteria he kills when he takes a bath. The argument is not that rights for cows means bacteria must be allowed to vote; we all recognize the need to draw lines and we should all recognize the fallacy of the argument that it is impossible to draw a line in a continuum (an acorn is not a tree even if it does by imperceptible degrees become one). The real objection to the animal rightists' view is that since we all recognize a hierarchy of species, why is it somehow morally compelling to draw the line between bacteria and insects, say, but morally reprehensible to draw a line between humans and all others?

In honesty, we should acknowledge, too, that as humans we are a part of the natural world, a world that is in a constant state of tension and competing interests between species. We should resist the temptation of viewing the natural world as a blissful, magical kingdom, save only for man, a clod with heavy boots trampling the flowers. The "sentient, purposeful" creatures of the wild lead difficult, violent, parasitized and short lives. Man's exploitation of animals for his own survival is hardly a perverse departure from the natural order. And, in the context of putting man's actions in perspective, those who oppose his exploitation of animals for research should ponder the 13.5 million dogs and cats that are put down in the United States each year for no reason whatsoever except that no one will take them as pets. (In Britain, ten times as many are killed in this way as in laboratory experiments — see

page 527.)

None of this implies that human beings can treat animals as they choose. Perversion — and corruption of human values of compassion — undeniably comes from pointless cruelty to animals. That we recognize a moral obligation to treat animals with compassion and to respect their undeniable interests is evident in laws prohibiting cruelty and requiring the preservation of species from extinction. But there are simply no consistent or universal principles that imbue animals with "rights" as exercised by humans. Individuals may of course differ in their personal tastes, and there is nothing wrong with one's personal compassion outweighing any desire to eat meat, for example. But we should all eschew the self-righteous delusion that our tastes are universal moral truths. □

Science for the young

British primary schools have made the teaching of science sensible. Now they must make it exciting.

How would you find out whether a ball that bounces best on one surface bounces best on all surfaces? How would you tell why a snail will not cross a ring of salty water? These are some of the questions that have been put to 11-year-olds in British primary schools by a group of investigators from Chelsea College, London, and the University of Leeds, as part of a survey of the science skills of British children sponsored by the Assessment of Performance Unit of the Department of Education and Science. The outcome, surprisingly, is encouraging. In these and other questions like them, roughly half of a representative sample of children (girls as well as boys) provided a reasonably convincing account of how they would set about the tasks assigned. Surrounding a snail alternatively with distilled water and brine, for example, was widely recommended as a way of understanding its behaviour. That about half of 11-year-olds grasped the essence of the problem is, in all charity, as much a triumph for British schools as the fact that half failed to get the point is a proof of failure. That few also pointed out that an attempt should be made, when repeating the experiment, to control for other variables is hardly a cause for shame, at 11.

That is the bright side of the coin. It suggests that in the past decade, a substantial number of teachers in British primary schools have abandoned the once-popular belief that the innocence of childhood observation coupled with tender unbridled imagination together constitute an approach to the understanding of the natural world distinct from science but just as valid. It seems that the days have gone when primary school teachers believed that if a child were encouraged to exercise his natural gifts for long enough, he would be discovered to have rediscovered Newton's laws or, better still, some alternative to them. The most encouraging aspect of the survey is the evident concern of teachers that young children should be able to tackle simple problems constructively, and by methods that will yield results. That children appear less able to provide general statements explaining disparate observations is not nearly as shocking as the report suggests. A frustration of childhood, only partly compensated for by imaginativeness, is that useful hypotheses derive more from knowledge than observation.

So why should young children bother with science? The most obvious defect of the investigations so far is that they say little about motivation except that, at least at eleven, girls are just as interested as boys. But everyday experience with young children suggests a zeal and obsessiveness with natural phenomena of a kind much admired in able graduate students, and which is not well catered for in primary schools. Separating salt from sand is still one of the standard experiments hardly likely to do much to satisfy (and thus stimulate) curiosity about the way in which the Universe is put together. No doubt the authors of the survey would point out that science in British primary schools has come a long way and is now going in the right direction. But there is a long way to go, even in the fortunate absence (in British schools) of controversies about such issues as creationism. □

UK research funds

Council's blunder leaves earth sciences stranded

AN administrative miscalculation means that the UK Natural Environment Research Council (NERC) and the scientific community that it supports are facing a severe shortage of funds for new research next year as a result of errors in forecasting within the council's university support section. At a meeting last week, the council decided to scrape together sufficient money so that at least some new projects can be started in 1984. Others already approved by the council's committees will have to wait until the beginning of 1985 unless further funds can be found in the meantime.

NERC spends just over £4 million a year on university research in the aquatic, atmospheric and solid earth sciences, while most of its £87 million budget is spent in its own research institutes, currently under pressure as a result of cuts in spending on "commissioned research" from government departments. The shortage of cash for new research in universities became clear only in October. Research grants usually last four or five years so that, of the total cost, typically 20 per cent would fall due in the first year and 30 per cent in each of the two subsequent years. Thus in any year, most of the £4 million spent on university research is already committed.

NERC seems now to have realized that awards made in previous years have led to a current over-commitment of about 20 per cent, so that the £800,000 or so that would normally have been used to begin new projects is not available. Those present at the October grant committee meetings were told that the projects that they were in the process of approving could not be started until 1 January 1985. (The committees meet in October and March and typically approve 140 or so grants a year — about one-third of the total applications.)

The exact cause of the problem is unclear. One component (although not a major one) is that the Committee of Vice-Chancellors and Principals (CVCP) reached a pay settlement earlier this year that had a considerable impact on NERC's finances. A research grant covers employment of research staff, purchase of equipment and travel and subsistence. For most grants, the research staff represent the major cost burden, and they usually occupy the lower rungs of university pay scales. In April this year, CVCP agreed to a pay settlement that amounted to about 4.6 per cent, but because the lowest rung of the relevant pay scale was abolished, the cost of the cheapest research assistant increased by about 13 per cent.

Such a factor can hardly explain a 20 per

cent over-commitment, however. When asked to explain the origin of the problem, the council's chairman, Sir Hermann Bondi, said that "the system that was being operated by the council's university support section to forecast the effect of inflation proved inadequate". When the older grants that are still current were begun (1980), inflation was running at 15.4 per cent, but it has since fallen steeply and is now running at about 5 per cent. In these circumstances, errors in inflation forecasting would be expected to be in the council's favour, so that the conclusion that somebody within the council has been incompetent seems hard to escape.

At a meeting last week, the council decided that just over £100,000 should be redirected to permit the start of the "most urgent" grants in 1984. Thus, of the 61 grants that were approved in October, 20 or so will after all be allowed to start from 1 January 1984. Bondi was at pains to emphasize that the flow of money to universities in 1984-85 would be greater than for many years and, furthermore, that the council would be trying to find more funds from within its budget. It will not be asking the Advisory Board for the Research Councils for help.

Philip Campbell

UK agricultural research

Plans for 250 jobs to go

THE Agricultural and Food Research Council (AFRC) has now completed its strategic plan, intended as an adaptation to the expected substantial drop in its share of the science budget over the next few years. The plan, which has not yet been ratified, will be discussed next week at a meeting of AFRC's governing council.

As AFRC's secretary, Dr Ralph Riley, promised two weeks ago, the Agricultural Research Service will suffer "rather severe damage". The strategic plan suggests that all arable crop research should be moved to Rothamsted Experimental Station, in Hertfordshire. The work of the Weed Research Organization, in Oxfordshire, and the Long Ashton Research Station is to be centralized on one site, as is the research undertaken by the National Institute for Research in Dairying, near Reading, and the Grassland Research Institute in Sussex. Somewhat more tentatively, AFRC will consider moving additional animal research to the Institute of Animal Physiology in Cambridge. There are also plans to alter the remit of the Welsh Plant

Words from space

NATURE is able today to report the exciting news that there is life — of a kind — in outer space. Through the marvels of modern communications, President Ronald Reagan, Chancellor Helmut Kohl of West Germany, your correspondent and a few other European journalists were able on Monday to direct questions to a tiled, delta-shaped object circling the Earth, from which certain four-limbed silver-suited organisms responded apparently according to some preprogrammed instructions. Here is a sample of the conversation:

"Hello, this is London [we were instructed to say that] Robert Walgate from the science journal *Nature*. Is it possible for you to tell us yet of any preliminary results that you have from your experiments on board?"

Ulf Merbold replies "Yes — the Grille spectrometer [a Franco-Belgian experiment] measured very interesting things — we figured out that the . . . atmosphere contains a lot of microconstituents like ozone, carbon dioxide and [undecipherable]. And we got very accurate information on the concentration of these gases in all the different layers of the atmosphere. That is important, to develop a model of the atmosphere which will then predict, for instance, for how many more years we can pump carbon dioxide into the atmosphere [undecipherable] impact on the climate."

After a few more questions communications failed, and inserted a random chunk of soap opera in which a child chanted appropriately "Where are we?" as if disembodied from his transmitter, followed by a picture of the Earth with a satellite on it. Then someone back on Earth said "Come again another day". We may. On the other hand we may not.

Robert Walgate

Breeding Station to encourage more work on upland farming.

The other general themes contained in the plan have been known for some time. AFRC intends to expand its work in food research: the Rowett Research Institute in Aberdeen is scheduled to expand its work to encompass research on trace elements and nutritional disorders and other institutes may also benefit from this programme.

Job losses in the Agricultural Research Service as a whole are likely to be even higher than was expected only two weeks ago. By the latest account, 250 jobs will go before April. Institute directors have been asked to offer pay in lieu of notice.

Two weeks ago, on the occasion of the publication of the ARC annual report, Dr Riley said that the amount spent on agricultural research in Britain is roughly the same as that spent by 200 high-energy physicists. "I hope that those who make decisions between such different subjects know what they are doing." Tim Beardsley

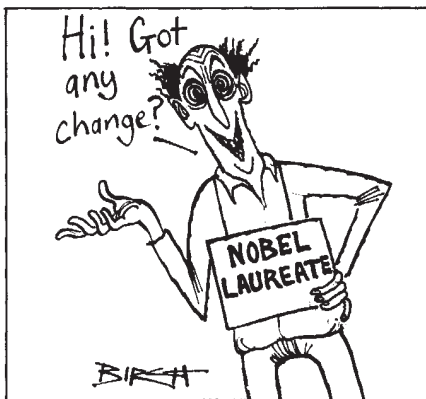
World Unification Church

Mixed motives at conclave

Chicago

UNDAUNTED by 12 years of intermittent controversy, distinguished academics continue to flock to the annual conference on "the unity of the sciences" sponsored by Reverend Sun Myung Moon's Unification Church. This year's conference, held here last week, heard the Nobel economist F.A. Hayek explain why socialism is the product of a "demonstrable philosophical error". Another Nobel laureate, physicist Eugene Wigner, was the conference chairman. And Robert Mulliken, who won the Nobel prize for chemistry in 1966, also attended.

The presence of three Nobel laureates was something of a coup for the Unification Church, which has spent millions of dollars on efforts to gain a foothold in the academic community. The annual conferences — organized by the New York-based International Cultural Foundation — have been the church's principal vehicle. But the church also supports a think-tank, the Washington Institute for Values in Public Policy, an organization called the Professors World Peace Academy and an academic publishing company, Paragon



House. Last year, the church began to award a biennial scientific prize. The first winner, Eugene Wigner, received \$200,000 (most of which he promptly gave away in the form of research grants).

Reverend Moon's lavish spending on the conferences has fuelled the suspicion of critics who believe the hidden agenda of the meetings is to win respectability for the organization. Last year, British Members of Parliament, complaining that the "Moonies" used coercion to make impressionable young recruits break off their studies, urged British academics to stay away. Conference organizer Morton Kaplan, professor of political science at Chicago University, conceded that many academics decline invitations to attend. But enough top-ranking scientists accept, and many become regular participants.

Why do they go? Scientists attending for the first time say they are impressed by the quality of the speakers and organizers. Luminaries on the organizing committee

for the Chicago conference included Alvin Weinberg, director of the Oak Ridge Institute for Energy Analysis; Karl Pribram, Stanford professor of neuroscience; Frederick Seitz, former president of the US National Academy of Sciences and president of Rockefeller University; Fred Singer, professor of environmental sciences at the University of Virginia and Nobuyuki Fakuda, president of Japan's University of Tsukuba.

Thomas Donahue, chairman of the National Academy of Sciences' space science board, attending for the first time, said he decided to do so because all the speakers in his session — on atmospheric science — were first class scientists. They included William Kellogg and Ralph Cicerone of the National Center for Atmospheric Research in Boulder, Colorado. But Donahue said he doubted whether he would attend again because the discussions accomplished little.

A small minority of those who attend are not aware of the link between the International Cultural Foundation and the Unification Church. A British scientist, Andrew Watson of the Marine Biological Association in Plymouth, was due to take the chair at a conference session but withdrew at the last moment when he discovered the event was sponsored by the Moonies. And Paul Sigmund, director of Princeton's University Latin American studies programme, said he found out about the Unification Church connection only at a planning meeting six weeks after the original invitation.

Most of the 300 participants at the conference were in no doubt about the identity of the sponsor. They explained their presence by describing the event as a rare opportunity to meet fellow academics from a very wide range of disciplines and countries. Kenneth Mellanby, former director of Britain's Monk's Wood Experimental Station and next year's conference chairman, said the conferences were not intended to enable scientists to learn new facts about their own discipline, but to encourage discussions across disciplines and focus on pressing world problems.

All the participants emphasized that the conference organizers made no attempt to influence the content of the papers delivered. Even so, the Chicago conference was not just another academic meeting. For one thing, the Reverend Moon's personal influence was considerable. Moon chooses the title of the conferences and delivers the opening speech. The title is usually grandiloquent and vague, this year "Absolute Values and the New Cultural Revolution". According to Kaplan, the title has little or no practical effect on the programme, although one aspect of Moonie theology, the notion that knowledge can be reduced to a few

unifying principles, is commonly echoed in a discussion group on the philosophy of science and the unity of the disciplines. Reverend Moon's opening address, too, was uncomfortably political for a supposedly neutral scientific gathering. Speaking with passion against communism, he claimed that the KGB and the Japanese Communist Party were organizing attempts to "deprogramme" members of the church.

Finally, the church makes little secret of its desire to use the conferences to make influential friends in academic life. In Chicago, a booth at the conference distributed literature explaining Moon's pending Supreme Court appeal against his conviction for tax evasion. And many of those who attend the meetings are later invited to an all-expenses-paid seminar at which the principles of Moonie theology are explained. In 1983, such seminars were held in Italy, the Bahamas, Thailand and Argentina.

The Chicago conference, smaller than its predecessors, is expected to cost about \$600,000. Participants' air fares and hotel bills are fully paid, and speakers receive a fee of \$500 and another \$500 if they revise their papers for publication. Most speakers nevertheless scoff at the suggestion that they attend for the money. **Peter David**

Institute settles lawsuit

THE Linus Pauling Institute of Science and Medicine in Palo Alto, California, has settled yet another lawsuit brought by a former employee who claimed to have been dismissed from a tenured research position. Dr Fred Westall, who worked at the institute in 1978 and 1979, had accused Pauling of fraud, breach of contract and slander. Under the settlement agreement, none of the parties may discuss the terms of the settlement.

According to court documents filed by Westall, he was offered the research position in February 1978. Westall claimed that in May 1979 he was fired without warning and without a hearing on any charge. Earlier this year, the Pauling Institute settled a suit brought by Dr Arthur Robinson, co-founder and former president of the institute, for \$575,000.

Westall had been a graduate student of Robinson at the University of California at San Diego and had joined the Pauling Institute at his recommendation. His termination at the Pauling Institute came shortly after Robinson's.

Pauling said last week that Westall "was a student of Robinson's" and that with Robinson gone, he believed Westall would not want to stay. "He never worked at the institute", Pauling said, but, citing the secrecy order on the settlement, refused to elaborate. Pauling also said that he had personally contributed "a considerable amount" of the money that went to pay Robinson's and Westall's settlements.

Stephen Budiansky

UK environment

Conservation plans go awry

THE Wildlife and Countryside Act, introduced two years ago, has run into serious trouble. British conservation groups are complaining that the act fails to provide adequate protection to places designated by the Nature Conservancy Council (NCC) as Sites of Special Scientific Interest (SSSIs).

The patchwork of 4,000 SSSIs in Britain is intended to be a key element of national conservation strategy, and owners of SSSIs are required to consult with NCC before undertaking any "potentially damaging operations" on them. But the 1981 act obliged NCC to "renotify" all owners and occupiers of existing SSSIs, specifying what operations might be potentially damaging. This process usually entails resurveying and has been averaging 20 man-days per site, with the result that NCC cannot comply with its obligations. Renotification is unlikely to be completed before the end of 1986. Earlier this year, a review of NCC by the Rayner unit, the government's cost-cutting team, reached the unusual conclusion that NCC needed more staff to carry out the task.

The snag is that until SSSIs have been renotified, they have no statutory protection from their owners. Many of NCC's manpower resources have therefore been diverted from designating new SSSIs to coping with threats to existing SSSIs.

Apart from the problems caused by the delay in notifying owners, critics of the act say that the controls granted after notification are also inadequate. When an owner notifies NCC of plans to carry out a "potentially damaging operation", NCC has three months in which to persuade him to modify the plan. If persuasion does not work, NCC may enter into a management agreement — an annual payment from NCC to compensate for loss of profits.

One consequence is that commercial companies have sprung up specializing in the purchase of SSSIs, opting for management agreements (and compensation) when development is denied. In one management agreement, NCC is negotiating to pay £20,000 a year for the next 60 years to the Marquess of Salisbury in return for his agreeing not to replant an ancient deciduous wood with conifers. The public is not at present allowed access to the site.

Although if an owner of an SSSI refuses to cooperate by means of a management agreement, NCC can as a last resort apply to the Secretary of State for the Environment for an order prohibiting the proposed development, these appeals (usually granted) are also a cause for complaint.

One recent case in Yorkshire exposed serious differences between the department and NCC. The underlying issues are the criteria for designation. The NCC criteria include scientific value, more subjective amenity value and consider-

ations such as the spacing of sites at suitable intervals so as to provide an adequate habitat for many less common species. NCC's request that the Yorkshire site should be protected was turned down by the Secretary of State on the grounds that the national importance of the site had not been demonstrated. The crisis may be averted, as an alternative site for development has been found. But the underlying issue has still not been resolved between NCC and the department.

NCC's estimates of the alarming rate of loss of wildlife habitats since 1949 have been publicized by Wildlife Link, a coordinating body for conservation groups. Ninety-five per cent of lowland rich herb meadows, 80 per cent of chalk and limestone grassland and 70 per cent of

acidic heaths head the league table of habitat losses. Dr Derek Ratcliffe, NCC's chief scientist, says in a draft document that "the rate and scale of loss is so alarming that the first objective of conservation must be to stem the tide".

Government ministers have recently defended the existing act, saying that voluntary agreements avoid the "conflict and bitterness" that blanket protection would entail, while NCC describes owners who have refused to cooperate on conservation agreements as "isolated mavericks". The question now is whether the isolated mavericks will become the norm. Despite the ministers' assurances, there is some serious concern in government circles about whether the act will provide the protection that the public demands. But another act so soon after the last one would be unlikely, and it will be at least another 18 months before a judgement is made.

Tim Beardsley

Finnish environment

New ministry's teething problems

FINLAND is planning a massive modification of its environmental legislation over the next ten years. At present, the laws of environmental protection are a confused collection of *ad hoc* acts introduced piecemeal to cope with specific problems. The need for a legislative review was underlined by the creation of a new environmental ministry on 1 October 1983 to deal with problems of housing and land use as well as of environmental protection.

The resettlement of upwards of 250,000 inhabitants of the territories ceded to the Soviet Union after the Second World War and the relatively late industrialization of the country (which led to the movement of over a million people from rural to urban areas) have put considerable pressure on housing and land use. More recently, there has been growing anxiety about acid rain. There is strong evidence that the acid rain acts synergistically with extremely low winter temperatures to cause considerable damage to the conifer forests which are Finland's major natural resource and ultimately the source of 45 per cent of Finnish exports. Aerial spraying with lime is impracticable in Finland — the areas involved are too great, there is little indigenous limestone and the cost is prohibitive. A recent survey has shown that one third of the 300,000 tonnes of sulphur precipitated on Finland each year originates from Finnish chimneys, and new standards on sulphur emission which will be included in a forthcoming bill on atmospheric pollution have been condemned by some ecologists as too lenient.

In all, it has taken twelve years to get the environmental ministry established. This is partly the result of Finnish politics, with a succession of coalition governments seeking consensus, and of vigorous opposition to the new ministry from within the existing

bureaucratic structure. Opposition came mainly from the Ministry of Social Affairs and Health, whose National Board of Health considers that its own municipal health boards already cover environmental matters. The new ministry hopes to use the network of local environmental health officers and inspectors operated by the Municipal Health Boards. This, some health officials fear, could lead to the boards in effect subsidizing the new ministry.

According to Olli Ojala, general director of the Department of Environmental Protection and Nature Conservation of the Environmental Ministry, it is precisely at this municipal level that environmental issues bite most keenly. Although Finland is divided into 12 provinces, provincial administrations are appointed from Helsinki and there is no elected level of government between the municipalities and the central government. As a result, the municipal level has gained considerable strength, and conflicts can arise when, for example, a pulp mill is both the largest source of municipal taxes and the major potential pollution hazard in an area.

This situation seems to have been fully recognized by Finland's newly emergent "Green" movement, which has gained strength during the public discussion of a possible fifth nuclear power station. In the general election last March, two Greens were elected to the Diet (sitting as independents as they have no formal party organization). Whether a formal party structure should be established was, indeed, the main topic of discussion of the 300 participants in the Green movement's congress at the end of October. They decided to concentrate on building up local support in preparation for next year's municipal elections.

Vera Rich

European biotechnology

Surplus food into feedstock

OUT of the political and economic gloom over the Athens summit earlier this week, where European Prime Ministers were attempting to thrash out a solution to the European Community's burgeoning "budget problem", officials were hoping for a little "green light" for biotechnology. The heads of government at Athens had before them a small but surprisingly punchy document outlining why Europe needs a 200 million European Currency Unit (roughly \$200 million) five-year programme in biotechnology research and development.

According to one official of the European Commission, research commissioner Etienne Davignon had to "bang heads together" to get agreement on the document, which crosses the interests of many previously isolated Brussels directorates. Notable among them is the agriculture directorate, the elephant which manages the Common Agricultural Policy (CAP) and spends 60 per cent of the EEC's funds accumulating agricultural surpluses; this directorate has admitted, for the first time, that biotechnology might have something to say about the development of new or better crops, the use and pricing of surpluses and the best management of land for biotechnological industry as well as food. This alone was a Brussels revolution, said the official. Moreover it could help to defuse conflict over the apparently iniquitous distribution of the CAP funds.

At Athens, the European Commission was not seeking immediate commitment to spend — just a nod that would indicate that their study of a possible large-scale biotechnology programme could go ahead. Beyond that, the plans would be to make a firm programme proposal for decision by a council of European ministers sometime between January and June, during the six months period of French presidency of the council. France is interested in creating a "scientific and technological space" in Europe, and would help the programme through, Commission officials believe.

According to the Commission document, "the Community is being outspent by the United States by a factor of 2:1 in public sector research, and more in industry; and 'outplanned' by Japan, which has for more than ten years been elaborating a coherently planned approach to developments in the life sciences and their industrial and medical applications. . ."

It quotes a US report prepared for the Office of Science and Technology Policy of the White House which said of Western Europe that the biggest obstacles to growth in the world biotechnology market were "the lack of qualified scientists and engineers (particularly in process and purification technologies), inadequate industry/university cooperation, and

belated and insufficient research and development funding by industry and government".

The Commission ascribes European weakness to fragmentation of efforts into groups and programmes too small for the problems tackled, scarcity of qualified staff and — at the Community level — regulatory, trade and other barriers to European cooperation. Biotechnology also needs extensive support from data banks, collections of organisms, technical facilities and patent counselling, and development requires "clear regulatory regimes at all stages from laboratory development and testing through marketing to post-market monitoring".

French computers

Double-deal at Orsay

EVEN if French academic computing power is behind that of the United Kingdom and West Germany (see *Nature* 304, 298; 1983), things may be looking up. After two years of often bitter negotiations, the biggest scientific campus in France — the Université de Paris Sud at Orsay — has at last been granted a new computer. Or, to be exact, two new computers.

This is the essence of a compromise now reached which leaves the university, government and the French computer company Bull all moderately happy. The university gets the American machine it wants and a Bull as well, but it must offer its expertise to the French company through a joint two-year research programme.

The two-year problem at the Orsay computer centre, Paris Sud Informatique (PSI), was that the existing machine (a Sperry Univac) was both old and saturated by the demand. The natural replacement was a new Univac, but the Mitterrand administration was committed to the development of French industry. Buy Bull, said the administration.

Bull (which markets French-built Honeywells) has no experience of scientific computing, said PSI — and anyway none of the extensive PSI software would run on a Bull: to buy Bull would have set many Orsay research programmes back by years. The result was two years of deadlock, punctuated by demonstrations and threats of resignation.

The happy conclusion, however, gives PSI its Univac (an 8-million instructions per second (8 Mips) 1100/91) and a 3 Mips Bull DPS 8/70 with the MULTICS multiplexing and time-sharing system developed for Honeywell at the Massachusetts Institute of Technology. The bill is FF 60 million (£5 million) to be divided between the ministries of education and research and industry. In return, Orsay must deliver

The Commission's programme would cover research and training, the concentration of national and other biotechnology policies, and acceptable ways of using agricultural outputs for industrial use (which is primarily a matter of reducing the support price for certain amounts of surplus). Research and training would be divided into "horizontal" activities — meaning pre-competitive basic research, and "specific actions" aimed at Community problems such as health care.

Of these programme elements, research, development and training would cost most (106 million ECU). Informal soundings of governments had yielded a positive response to Commission ideas, said an official on Monday, but strong reservations over the cost of the research, development and training element.

Robert Walgate

42 man-months of research and development, matched by 21 man-months from Bull, aimed at helping Bull develop expertise at scientific computing.

But the agreement "will be a failure" if it stops after just two years, said the president of the PSI users' committee on Monday. "We have a lot of scientists at Orsay who make very heavy demands on computing", said M. Granet, "and we want to help Bull into this market." Initially the work would involve mostly matters of software and networking, but there are rumours of Bull developing a big, all-French scientific computer. According to a Bull spokesman in Paris, there is indeed a plan to build a big vectorial machine — like a Cray — for the French military, and this could have uses in certain sciences requiring parallel computation. According to M. Granet, Orsay could become "a platform" for trying out any new French machine.

Meanwhile, PSI awaits its new machines, and in 1986 a promised upgrade of the DPS 8/70 to a 9 Mips DPS 88. It also expects to link the machines with the Cray-1 now installed at the nearby Ecole Polytechnique, initially using coaxial cables running at 2 Mbits per second and — perhaps within two years — fibre optics running at 34 Mbits per second.

And back at the ministry of research and industry, there is relief that the Orsay affair is over, and hope that a general protocol for computer purchase by universities, research councils and so on will be ready before the end of this year. The object of the protocol would be to establish a framework agreement — in particular involving research assistance to Bull, which has previously had few links with universities — which would avoid the kind of detailed institution-by-institution wrangling experienced at Orsay. It may not be officially published, a ministry spokesman said.

Robert Walgate

Australian telecommunications

Private sector at arms' length

Canberra

THE Labour Government has been busily reversing the free-market bent of Mr Malcolm Fraser's policy on telecommunications in order to keep it in the public sector. The latest policy reversal is the decision on 15 November to retain full ownership of Aussat Pty Ltd, the company set up to own and operate the proposed domestic communications satellite system, Aussat, due to be launched in July and October 1985.

This decision has put paid to any hope of private sector equity in the satellite. The previous government's plan to sell off 49 per cent of the equity, chiefly to the commercial television networks, as soon as practicable (see *Nature* 297, 449; 1982) was provisionally endorsed by the new government in May this year. But it seems the government has now given in to pressure from the Australian Telecommunications Employees' Association which was against private ownership. The decreased deficit in the August budget has helped.

The government has also given Telecom Australia the option of taking up 25 per cent of Aussat's shares, which will strengthen Telecom's monopoly position by giving it participation in board decisions. Ironically, the satellite was originally seen as the first opportunity to set up communications and information services in competition with the public carrier, because by law Telecom has a monopoly over terrestrial microwave links, but this possibility now seems increasingly remote.

Aussat, operating as a self-contained commercial venture, would in theory lease its transponders to the highest bidder, so there may yet be private participation. But competition is likely to be closely regulated as a result of the referral by the Communications Minister (Mr Michael Duffy) to the Australian Broadcasting Tribunal of the decision on the allocation of the four high-powered transponders on the second satellite. (High-power transponders on the first satellite have already been allocated to the Australian Broadcasting Corporation for its homestead and community broadcast services, which will beam programmes directly to remote homes that have a 1-m reception dish.) Aussat's government and Telecom equity is likely to be entrenched by legislation to hinder another change of policy should the Labour Party again find itself in opposition.

In the meantime, the minister has hinted darkly that the first satellite may have been "oversold" in respect of services for the outback. While it may be possible for listeners to receive School of the Air or other city broadcasts, they will not be able to transmit replies without installing remote Earth stations at a cost of \$A10,000–15,000. In addition, direct satellite broadcasting by commercial

stations is no longer an option, that right being reserved exclusively for the Australian Broadcasting Corporation. To prevent regional networks from going out of business, commercial stations will have to transmit to regional stations via satellite-scrambled signals which will be decoded by the local station and distributed through the network. Moreover, it now appears that the land-based digital radio concentrator system extending the terrestrial telephone network may yet prove to be more economical.

Another change of policy — the second in two years — concerns videotext services. Plans for Telecom to introduce videotext came to nothing when the then Minister for

Communications, Mr Ian Sinclair, handed over the market to the private sector in October 1981. Telecom was, however, given the go-ahead from Mr Duffy in November to sell the British Prestel system and ultimately to accommodate other systems, after an Australian Science and Technology Council report recommended that Telecom should establish a national videotext service.

Two extensive reports into ways of involving the private sector in telecommunications have also fallen by the wayside, one on telecommunications services in Australia by a committee under the chairmanship of Mr Jim Davidson of Commonwealth Industrial Gases and the second on cable television by Mr David Jones, who is the present chairman of the Australian Broadcasting Tribunal.

Vimala Sarma

Laboratory animals

Huxley attacks "animal rightists"

IN a stout defence of laboratory animal experiments, Sir Andrew Huxley, president of the Royal Society, last week let slip that he personally countersigns 1,500 applications a year by researchers to the Home Office — and that he sends about 100 of them back for amendment before adding his signature. This is not merely a proof that Huxley ("I do not use a rubber stamp") is a most meticulous holder of his office but also a reason why the Royal Society welcomes the British Government's plan to introduce new legislation on animal experiments, either in the autumn of 1984 or a year later.

Under the legislation, intended to conform with the Council of Europe's draft convention on animal experiments, Huxley (or his successor) and the presidents of the medical colleges will be relieved of their statutory responsibility to countersign applications for licences for experiments with animals. Huxley emphasized last week that most of the applications sent back for amendment had provided inadequate — "sometimes perfunctory" — information, and that only "once or twice" had Huxley doubted their ethical justification.

Huxley, in his anniversary address on 30 November, also complained at the disruptive activities of "groups of terrorist thugs" from organizations such as the Animal Liberation Front, who break into laboratories and abuse individual scientists, not only verbally. He went on to suggest that public opinion is often swayed by television and other "media", "perhaps to increase the circulation of a newspaper".

The essence of Huxley's argument last week was an echo of Edmund Burke attributed to Professor Bernard Williams, provost of King's College, Cambridge, and a member of the society's ethical working party: is the question whether "animals

have rights, or is it merely that humans have duties towards animals?" Huxley pointed to the difficulty of assessing the pain experienced even by other human beings, let alone animals, asked what importance can be attached to the painless killing of purpose-bred laboratory animals that otherwise would not have lived and pointed out that the Royal Society for the Prevention of Cruelty to Animals kills 100,000 unwanted pet dogs and cats each year, ten times as many as are used in laboratories.

The Royal Society's view on future legislation, accepted by the British Government, is that there is no need to relax the "pain condition" which at present requires that animals suffering "severe pain which is likely to endure" should be killed immediately after an experiment except under special licence, even though the European convention will be less restrictive. According to Huxley, the explanation is that the commitment of the West German constitution to untrammelled scientific research accounts for the provision in the draft convention that such experiments might be permitted if judged to be of "exceptional importance".

In Huxley's opinion, while "urgent human need" (such as the need to understand and treat a newly emerged disease) might justify exceptions to the pain condition, the "advancement of knowledge" is not a sufficient reason so that "there are circumstances in which it is legitimate to restrict the freedom of scientific investigation". Huxley also said that in his opinion, the use of local ethical committees as a means of licensing experiments with animals, while successful in countries such as Sweden, was an alternative to the "well-trying" British system based on the inspection of laboratories by the Home Office, and should not replace it.

John Maddox

Scientific publishing

Academic backs down on books

EUROPEAN publishers were last week astounded by the announcement by Academic Press London (APL) that it is to make redundant more than half of its 247-strong workforce. The recent history of APL has been troubled (see *Nature* 303, 192; 1983) and changes were expected, but the scale of job losses came as a shock to both staff and the publishing community. Most significantly APL is to shut down its book production department entirely, with the loss of some 30 jobs. The new warehouse opposite the company's offices in Camden, North London, opened only a few months ago, will also close. From the middle of 1984 almost all book production and the fulfilment of orders for books will be carried out in the United States. The editorial department in London is largely unscathed, however, and the very profitable journals department is not affected.

APL is a wholly-owned subsidiary of Academic Press in the United States, which in turn is a subsidiary of Harcourt Brace Jovanovich (HBJ), a Fortune 500 company with diverse interests. The redundancies in London are part of a large-scale reorganization of the company as a whole, implicit in which is the curtailment of the output of scholarly books because of "very high overheads and price resistance in the

marketplace". By contrast, a memorandum of 21 October to senior staff and agents from Mr William Jovanovich, president and chief executive of HBJ, makes clear that "the number of . . . journals can be increased".

While a number of academic publishers have recently experienced difficulties because of steeply rising costs, many of APL's problems have undoubtedly been self-inflicted or imposed by the American parent company. Print-runs, reflecting estimated demand, have in the past been over-optimistic which has resulted in large amounts of stock being written off. And, because of a pricing policy described as "sheer incompetence" by one member of staff, APL had to sell at a very narrow margin books produced by the company in the United States. The decision last year to lease Joel House, in Camden, as a warehouse seems also to have been financially disastrous.

The effects of a reduced output of scholarly books by Academic Press as a whole will not be felt immediately. One long-term possibility is that worthy but unprofitable books will be even less likely to be published, another that scientists will turn increasingly to journals.

Tim Lincoln

Collective phenomena

Absent Soviet friends

Stockholm

THE Sixth International Conference on Collective Phenomena last week cast light on the problem of scientific "refusniks" in the Soviet Union. Although many Western scientists have in the past ten years visited the regular Sunday seminars organized by the refusniks (Jewish scientists denied exit visas but dismissed from their scientific posts), and have participated in the three conferences on collective phenomena which the Soviet authorities have grudgingly allowed to take place, such participation has been more an act of solidarity with oppressed colleagues than a serious scientific effort. (The first and fifth conferences were prevented by *force majeure*.) For last week's conference, the first to be convened outside the Soviet Union, was a serious effort to put the continuing scientific work of refusniks into an academic context.

The Stockholm meeting focused on the continuing work of Jewish scientists in Moscow who for the past decade have been denied what is normally considered the basic prerequisite of scientific research, access to libraries and laboratories. Four refusnik papers were presented, by Viktor Brailovskii, Yakov Al'pert, Aleksandr Lerner and Naum Meiman, supported by papers on related themes by Western colleagues. This "supporting cast" contained

such eminent names as Paul Flory (United States) on the configuration of macromolecular chains in condensed phases, Andrew Sessler (United States) on collective motion in free electron lasers and Peter Whittle (Great Britain) on a socio-economic model showing collective effects.

The four refusniks papers were presented by colleagues working in related fields — not an ideal arrangement, since the ensuing discussion raised a number of unanswered questions. Thus Professor Al'pert's mathematical analysis of the absorption of ELF electromagnetic waves in the terrestrial magnetoplasma suggested an unexpectedly large value for the absorption under certain circumstances for which, owing to his limited possibilities for practical work, he provided no physical explanation. And Professor Lerner's paper "on the theory of mass behaviour in developed human communities" arrived without several pages of vital mathematics.

The fact that so many questions were left unanswered is evidence that, in spite of all difficulties, the refusnik scientists continue to do valuable theoretical work. This would seem to justify the exhortation that scientists on official trips to Moscow should continue to try, in spite of increasing difficulties, to visit the refusnik seminars.

Vera Rich

Creationism

Still in evidence in Texas

RELIGIOUS fundamentalists are shifting their efforts to local school boards, having largely failed in the courts and state legislatures to force the teaching of "creation science" in public schools. The current multiplicity of relatively low-key efforts seems, at least in some areas, to be paying off. In Columbus, Ohio, teachers are instructed to approach evolution as a "controversial" subject. In a Virginia county, teachers must preface a discussion of evolution with a reading from *Genesis*. And in the many southern states that select textbooks for statewide use, creationists have forced the elimination of references to evolution in biology texts.

The shift in tactics has come in the wake of some spectacular failures for the creationists in well-publicized court cases, notably the finding by a federal judge last year that an Arkansas law requiring equal treatment of evolution and "creationism" was an unconstitutional violation of the separation of church and state. "It cost Arkansas a bundle of money" to defend the statute, says Wayne Moyer, former executive director of the National Association of Biology Teachers and now a staff

Factor VIII cloning

GENETICS Institute, the Cambridge (Massachusetts) biotechnology corporation, claimed last week important progress towards the production of factor VIII, the blood-clotting agent in which haemophiliacs are deficient. The institute says that it should have been able to clone and express the gene for human factor VIII by next summer and to have a product in clinical trials roughly two years from now.

Some aspects of the work are being kept confidential for commercial reasons, but Dr Robert Kamen, scientific director at the institute, explained on Monday that the starting point has been the use of monoclonal antibodies for the isolation of fragments of factor VIII from pigs (in collaboration with Dr David Sass of the Mayo Clinic) and the use of microsequencer techniques (by Dr Rodney Hewitt of the institute) to derive partial amino acid sequences and then candidate gene probes. The probes have been used to identify substantial parts of the human gene, which turns out (as expected) to be on the human X-chromosome.

Precisely why Genetics Institute has made its announcement now is not entirely clear. Stock in the corporation is privately held, and there are no immediate plans for a public offering. The factor VIII programme is sustained by a research contract with Baxter Travenol Laboratories.

John Maddox

member of People for the American Way, a first amendment activist group; the lesson has not been lost on legislators in other states, Moyer says. Equal-time bills filed in Alabama, Mississippi, West Virginia and 21 other states have failed.

The place where creationists may be having the most success is in the state textbook adoption proceedings, particularly in Texas. Texas is now beginning its annual cycle of textbook selection, and last month the state's elected, and traditionally populist, Board of Education issued draft guidelines for publishers which hope to have their biology texts chosen as one of five that will be purchased for every school district in the state. The guidelines contain no reference to evolution; Charles Darwin is absent from a list of biologists who made important discoveries. The Texas board as early as 1974 issued administrative rules requiring textbooks that mention evolution to "clarify that the treatment is theoretical rather than factually verifiable" and to include a disclaimer that evolution is "only one of several"

explanations of human origins.

With 8 per cent of the national market at stake, Texas has become the focus of considerable concern for opponents of creationism. The Texas board routinely holds meetings with publishers to demand specific changes in the texts. Publishers frequently comply, saying that because it is not economical to produce a special Texas edition, the changes demanded by Texas determine textbook content throughout the country. In testimony before the board last month, People for the American Way produced several examples of changes ordered by the board and complied with by publishers. These typically consist of demands to preface such statements as "the earliest people lived on the Earth over 1 million years ago" with disclaimers such as "many scientists believe" or "according to scientific theory". A number of textbook publishers appear to have anticipated objections and adopted a policy of self-censorship, notably in purging the word "evolution".

Some more subtle forces may favour the

creationists at the local level as well. Mary Long, chairman of the science department at a high school in Austin, Texas, told a meeting sponsored by People for the American Way last month that a teacher who covers evolution in biology class and who receives a complaint from a parent is, in Texas, unsure of being backed by the school administrators. Most teachers, she said, prefer to avoid the hassle.

Despite the inroads that creationists appear to be making at the local level, Michelle Aldrich, who monitors the creationist efforts for the American Association for the Advancement of Science, says that "committees of correspondence" set up by scientists and educators to counter creationism have been "surprisingly successful" in attending local board meetings, presenting the scientific case and explaining the likely consequences if the matter goes to court. The school board in New York City recently went against the Texas tide by rejecting several biology texts because of their inadequate coverage of evolution.

Stephen Budiansky

UK biotechnology

Where there's muck . . .

ENCOURAGING signs of life in British biotechnology have been discovered by Biotechnology Investments Limited (BIL), the two-and-a-half-year-old company sponsored by the merchant bank N. M. Rothschild & Sons Limited. Since the end of May, when BIL's second annual report showed investments of \$34 million in about 30 companies (including six in the *Nature* index below), none of them in the United Kingdom, three essentially British investments have been made and a fourth is soon to follow.

The largest investment has been £5 million in Celltech last June; very recently \$0.6 million has been sunk in British sludge and this week the same sum is potted in ornamental plants.

The first of the two \$0.6 million investments has bought a 25 per cent interest in WMC Resource Recovery Limited, the ten-year-old brainchild of Victor Lawson, a consultant mechanical engineer. The patented process on which his Bristol-based company is built involves the anaerobic fermentation of domestic waste and sewage sludge to produce a fibrous material that can substitute for either wood pulp fibre or horticultural peat. As biotechnology goes, the process is steam-aged, but the results of four years of operation of a one-tenth scale plant has convinced BIL that the process works and that the products are commercially viable. The peat substitute has been extensively tested by Agricultural Research Council scientists and shown to be of good nutritive value, although some additions will be necessary for certain markets. Although heavy-metal content of the product is on the high side, the metals are mostly in slow-release form

so that only acceptable amounts end up in plants grown on the product.

WMC Resource Recovery Limited has recently negotiated its first firm order for a unit (in Spain) and expects others to follow. With the aid of UK government and EEC grants it will soon build a full-scale demonstration unit in Bristol. BIL's investment is designed to capitalize the company and provide it with a financial partner for a phase of anticipated growth.

BIL's investment in ornamental plants

is through Twyford Laboratories, in Somerset, whose parent company, International Plant Laboratories Inc. in Boston, has just raised some \$4 million from nine investors, of which BIL is one of four contributing \$0.6 million. Twyford Laboratories currently produce about 5 million plantlets a year by micropropagation, mostly for houses and offices in Europe but also have a growing interest in crop plants.

The third BIL investment in UK biotechnology is in a Cambridge-based diagnostics company; details will be announced in a week or two.

Peter Newmark

Nature index of biotechnology stocks

12-Month high	12-Month low	Company	Close previous month	Close 25 Nov.	Change
23 1/4	10 1/2	Biogen (Switzerland)	12 1/4	12	0
6 1/4	1 7/8	Bio-Logicals (Canada)	2 1/8	2	- 1/8
16 1/8	7 1/4	Bio-Response (USA)	11 3/4	11 3/4	0
19	10 7/8	Cetus (USA)	11 5/8	12 3/8	+ 3/4
15 1/2	6 3/4	Collaborative Research (USA)	9 1/2	8	-1 1/2
39 7/8	15	Damon (USA)	20 1/8	17 5/8	-2 1/2
34 1/4	16 3/8	Enzo-Biochem (USA)	24 1/4	24 1/4	0
18 7/8	8 3/8	Flow General (USA)	9 1/4	11 1/4	+ 2
49 3/4	25 7/8	Genentech (USA)	26 1/2	36 1/2	+ 10
17 3/4	7 7/8	Genetic Systems (USA)	9 1/4	10 5/8	+ 1 7/8
23 1/4	12 7/8	Genex (USA)	15 1/2	15	- 1/2
31	19 1/2	Hybritech (USA)	22 1/2	12 1/2	-1
22 1/4	12	Molecular Genetics (USA)	13 1/4	12 7/8	- 3/8
23 1/4	13	Monoclonal Antibodies (USA)	14 1/4	14 3/4	+ 1/2
73 1/2	42	Novo Industri A/S (Denmark)	66	65	-1
30 1/4	13 5/8	Pharmacia (Sweden)	25 1/8	21	-4 1/8

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. *Nature's* weighted index of biotechnology stocks stood at 196 on 25 November, compared with 203 a month earlier and 221 in September. Data from E.F. Hutton, Inc.

IOS closures

SIR — In a news item on the possible closure of Institute of Oceanographic Science laboratories (*Nature* 10 November, p.102), Philip Campbell belittles the status of Bidston Observatory by describing its work as being “in more applied areas such as tidal computations”. In a brief telephone conversation with Dr Campbell I may have cited the group which computes most of this country's (and Australia's) tide tables as one which is especially vulnerable to a move to Surrey, but it should not be taken to typify our work. Our research group on the tides of the ocean may be said to lead the world in this area of pure geophysics, while our solid-Earth-tide study group is the only one in Great Britain. We have well known expertise in numerical modelling of the tidal and wind-driven motions of the shallow seas surrounding this country, now being extended to include the nearby shelf-edge and deep ocean. Other researchers pursue advanced studies of such dynamic features as oceanic fronts, topographical waves and mean sea level. These activities are backed up by a strong in-house instrumentation team which specializes in seagoing monitoring techniques and tidal measuring equipment from the sea-shore to 5 km deep in the ocean. Last but not least, Bidston houses the data banking facility of the UK Marine Information and Advisory Service, an excellent marine library, and a local meteorological service.

D.E. CARTWRIGHT

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Garret Hardin

SIR — I must take umbrage at Robert Ubell's glib and prejudicial remarks concerning Garrett Hardin¹. Hardin's thrust does not bespeak “racism” nor “chauvinism”. Hardin's essential message is simply that our Earth is overpopulated and abused relative to available food and energy². In his classic essay *The Tragedy of the Commons* (not mentioned by Ubell), Hardin incisively analysed our dilemma with overpopulation. And, lest we forget his message, remember our numbers are now approaching 6,000 million whereas we stood at a mere 3,000 million or so a little over a decade ago. Will the coming generations really want to survive to see 12,000 million? 24,000 million? I doubt it for, as Kingsley Davis argued³, our population is even now too large to retain our past gains in quality of life.

L.H. WULLSTEIN

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BeliketheGerman

SIR — Regarding the continuing debate over nouns as adjectives, I have a simple proposal: English should follow the pattern used in German, where two or more words may be joined into a single word. This would resolve such conflicts as “base pair” versus “basepair”, and the latter would become standard wordusage. The question of which word modifies which is resolved by the orderconvention that wordfragments nearer the wordbeginning modify those farther along. Among the systemadvantages for *Nature*articlewriters would be journalarticlewordcountlimit-problemelimination, although optional parenthesesintroduction might facilitate clarityretention during, for example, this (((60-wordsentence))/(18-wordsentence)) reduction) process.

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Homology defined

SIR — We in comparative biology are subjected to more arguments over the meaning of the term homology. Mr Moore's reply (*Nature* 22 September, p.268) to Mr Hughes (*Nature* 24 March, p.706), iterates the thesis that the homology observed on a broad scale in systematic and evolutionary study is somehow different or less refined than the fine level homology which can be studied in extant forms.

For fine similarities of extant taxa, Mr Moore develops the concept of “paramology”. I find this proposition both cumbersome and naive. An unfortunate side effect of the sophistication of our scientific viewpoint is the propagation of jargon. If terms are introduced into the biological lexicon they should be metaphors for discrete phenomena or repeatable observations; not loosely constructed words with “derivatives”. Shall we soon see in the ethnology literature an ethnology describing a shared behaviour? Or a sub or ultraparamology denoting lower and higher levels of paramology?

A second, more fundamental, difficulty stems from Mr Moore's concept of homology. In its general definition homology pertains to sameness of structure, independent of the evolutionary history of that structure. I agree that the sameness reflected in Mr Moore's example of the similarity between tetrapod arms and wings seems different from the exacting similarities seen in the biochemical fine structure of extant organisms, but whether this observation represents an ontological difference is not clear. Homology, in a broad sense, operates at several levels, and as better molecular biological techniques are developed, Mr Moore's paramologous

structures will surely grade into homologies as discrete differences between paramologous structures are detected.

In practice homology is recognized on the basis of congruent distributions of attributes, and nonhomologous attributes are recognized by their noncongruence with this pattern. Amongst amniotes several clades are defined on the common presence of unique features. From this pattern we can recognize that a character, such as homeothermy, found in birds and mammals, is a nonhomology. Why? Because birds share more attributes with non-homeothermic crocodilians and lizards than they do with other homeotherms, that is, mammals. This indicates that the homeothermy of mammals and birds is not a general statement defining the level of the inclusive group of birds + crocodilians + lizards + mammals, hence this homeothermy represents two unique attributes rather than a homology.

The power of comparative anatomy in distinguishing patterns of homology is not new since it can be traced directly back to the pre-Darwinian typologists and perhaps even to Aristotle. This approach has resurfaced and been developed in recent years by practising systematists, somewhat independently, unfortunately, of other biologists. Admittedly eschewing a Hennigan view of the world, I wish to reiterate that homology is a comparative concept connoting sameness of structure, indicating group membership, and operating independently at several hierarchical levels — in the end Homology = Synapomorphy.

MARK A. NORELL

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Numerology

SIR — In the argument about numerology (*Nature* 7 July, p.11 and 1 September, p.8), both the numerologists and their critics miss the point. Being impressed by the near match of numbers does not make science, nor is it scientific simply to stress the remaining inaccuracy and complain of the lack of sense in the whole effort. This is a simple scientific problem: is there a phenomenon to be explained, or not?

This question can only be answered by making use of a statistical model which allows us to calculate accurately the probability of matching a number by a certain type of combination of specified numbers (such as 1, e or π) with an accuracy as good as the one actually obtained. If this should turn out to be well below the usual critical limits of 5×10^{-2} or 10^{-2} , the numerological result has to be taken seriously and cannot be dismissed by any non-mathematical reasoning. In cases not totally evident no numerologist can expect scientific credibility without first establishing such statistical evidence.

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Yrast phenomena in fashion

The invention of a word new in the past decade signals a resurgence of interest in nuclear physics. But this time the objectives, if attainable, could be important.

LEXICOGRAPHERS have been dismayed, but word-game players no doubt delighted, by the appearance on the scene of the word *yrast*, one of the rare contributions of the Swedish language to international usage. (The word is apparently the superlative of the adjective *yr*, which means "dizzy".) Its currency within the past decade reflects the interest which now centres on a recently unfashionable field of physics — the study of heavy nuclei by means of particle probes whose energies are modest by the standards set in high-energy physics laboratories, a few hundred MeV or thereabouts. Only last month, the general assembly of the European Science Foundation was congratulating itself on what had been accomplished, at least in the construction of specialized machines, in Europe in the past decade.

That the time is ripe for a resurgence of nuclear physics should not be a surprise. During the 1940s, interest in the properties of heavy nuclei was stimulated by the applications of nuclear fission then in vogue. (Thirty years ago, neutron cross-sections with heavy nuclei were still national secrets.) But the experience of that decade was itself sufficient to confirm what many people had expected, that the formal treatment of any but the simplest nuclei as systems of several interacting particles would be even more difficult than the calculation of the electronic structure of all but the simplest atoms.

Three developments have brought the field to life — the recognition that, with quarks and all that, nucleons are even more complicated but much more interesting than had been thought, heavy nuclei turn out to have previously unexpected but intriguing properties and there have been instrumental developments of great potential, both in the production of new kinds of probes for nuclear structure and in the measurement of what happens.

The experience of the past few years has shown that the rotational properties of heavy nuclei are arresting, well worth the invention of a word such as *yrast*. That a nucleus, being an object, should be capable of rotating about some axis is natural enough, while its small dimensions ensure that the rotation will be quantized, with permissible values of the total angular momentum determined by some integral quantum number and by Planck's constant.

But what if the nucleus is constitutionally asymmetrical, not spherical but, say,

mildly sausage-shaped (an oblate spheroid)? And what if the rotation of the nucleus as a whole is not around the remaining axis of symmetry but, rather, in a perpendicular direction? That nuclei in the ground state can be aspherical has been known for decades — isotopes of Yb or Hf are familiar examples. The underlying explanation lies in the Fermi statistics that control the collective behaviour of the nucleons.

The route to the recognition of the interest of such nuclei rotating about an axis which is not the surviving axis of symmetry involved the recognition that collisions between nuclei, perhaps to form a compound nucleus whose eventual product would be aspherical, could leave a nucleus in such a state that its rotational energy was comparable with the excitation energy separating one nucleonic state from another. A glance at one of the usual physics journals will show that people are regularly using rotating nuclei with angular momenta of 50, 80 or more quanta.

Last month's report to the European Science Foundation by the working group on nuclear physics was introduced by its chairman, Professor T. Mayer-Kuckuk from the University of Bonn, by means of an enthusiastic slide-show of the interest of these nuclei. One of his arguments is that a rotating nucleus, small though it may be, is not in principle very different from more familiar objects made of nuclear matter and rotating rapidly, the neutron stars which make pulsating stars for example. Certainly it seems to be on the cards that the further study of rotating nuclei will yield some information about the collective motion of particles in the much larger objects in the sky.

On the nuclear scale, it is already clear that the rotational state of a nucleus and the state of its internal nucleonic excitation are not independent. In classical language, the orbits of nucleons are affected by centrifugal and Coriolis forces (responsible, among other things, for cyclonic and anticyclonic patterns of terrestrial weather) caused by the rotation. On one view, that which models the internal conditions of a nucleus analogous to a superconducting state in which the motion of particles is truly collective, there is even a chance that sufficiently energetic rotation could cause a phase transition to a different state.

This view of the standard heavy nucleus as a model of collective motion in a relatively simple system, a hundred or two

of nucleons in which collective motion dominates, is new. It also accounts for the now fashionable search for collective vibrational states, some of which surprisingly consist simply of pure radial oscillations whose frequency points directly to the compressibility of nuclear matter. But collectivity is not all, and experience has shown that the rotation of some nuclei cannot be explained by a rotation of the object as a whole but rather requires that the angular momenta of individual nucleon orbits, considered separately, should be aligned.

In the years ahead, according to Mayer-Kuckuk, the field will be further enlivened by the further development of novel nuclear probes. The availability of anti-proton beams should make the nuclear equivalent of positronium possible. The use of strange kaons (strange particles in the quark sense) should make possible for the first time measurements of nucleon momenta near the centre of the nucleus. Relativistic ion beams (nowhere yet produced) may yield remarkably new kinds of energy transfer. One goal now firmly in people's minds is to use old-fashioned nuclear physics to test field theories of strong interactions hitherto thought accessible only by the high-energy physics people. Making a bag of quarks might even provide a microscopic simulation of the Big Bang, if ever there was such a thing.

The European Science Foundation's committee has been concerned not merely with what might be accomplished but with the arrangements that exist for keeping the field alive. Mayer-Kuckuk has made a survey of all facilities in Europe, with telephone and telex numbers as appropriate, together with a calculation of which countries in Europe support how many people in the field. West Germany has 20 nuclear physicists per million inhabitants, while Britain is a long way down the list with a mere eight.

On balance, however, it seems that the field will prosper and that the new word *yrast* will soon be on everybody's tongue. What can it mean? Half-way between a noun and an adjective to judge from its usage, the simplest occurrence (as in "*yrast trap*") refers to the case in which two states of a nucleus, at least one of which is excited, have different angular momenta but nearly equal energies, so that it becomes convenient to look for transitions from one to the other. Why not?

John Maddox

Deep Sea Drilling Project

Sediment drifts and intraplate tectonics in the North Atlantic

from the staff of Leg 94

ON 17 August 1983, the *Glomar Challenger* docked in St. John's, Newfoundland, with more than two miles of sediment cored from a climatically critical region of the North Atlantic Ocean. This ended a voyage begun 55 days earlier in Norfolk, Virginia, that took the *Challenger* to a transect of six sites from just north of the Azores at 37°N to west of Ireland at 53°N.

The central objective of Leg 94 was to obtain a north-south transect of high-quality drilled sections using the *Challenger's* hydraulic piston corer (HPC) and extended core barrel (XCB) techniques, in order to examine the response of the North Atlantic to climatic change over an expanded time scale spanning the past 5 to 6 Myr. All sites yielded continuous sedimentary sections with remarkable palaeomagnetic and biostratigraphic age control. Sediments were deposited at unusually high rates for the deep-sea (30–90 m/Myr) and will permit shore-based researchers over the next few years to trace oceanographic responses in the North Atlantic back into the Neogene. We confirmed a result reached on Leg 81 (Site 552): the first major shift from preglacial calcareous oozes to cyclically layered glacial and interglacial sediments occurred at roughly 2.4 Myr. Our results show that this change affects the entire east-central Atlantic south to 41°N; beyond there the glacial imprint dies out by 37°N.

Most immediate results of the drilling, however, came from secondary objectives aimed at understanding the history of the Feni and Gardar Drifts and the King's Trough tectonic complex north of the Azores.

Deep water circulation in the North Atlantic has had a profound effect on the nature and distribution of its sediments. Seismic reflection profiles over wide areas are characterized by anomalously thick sediment piles (sediment drifts) built by the flow of deep bottom water. Feni Drift on the western side of Rockall Trough and Gardar Drift on the eastern flank of the Mid-Atlantic Ridge are major bathymetric features that in places are over a kilometer thick and extend thousands of kilometers in a generally north-south direction. These major drifts are thought to have been built since the Paleogene, primarily by Norwegian Sea Overflow Water that spills over the Wyville Thompson and Iceland-Faroes Ridges. They were chosen because

high sedimentation rates were anticipated, but attracted interest from the sedimentological community because high-quality HPC and XCB sections could be studied to characterize bottom current deposits. Deep drilling at the sites might identify and date regional seismic reflectors through which to infer the history of bottom water circulation.

Sediment drifts are characteristically ornamented with large-scale sediment waves, generally tens of meters in ampli-

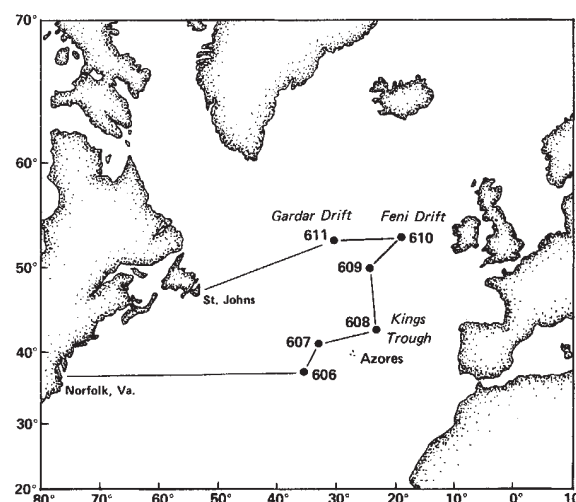
holes and give way to pre-glacial nannofossil oozes, marls and chalks. No primary structures that might be interpreted as due to the activity of bottom currents were observed. Gardar Drift sediments contained a greater terrigenous component than those on Feni Drift, but this was due to a greater input of ice-rafted debris from Iceland. Feni Drift sediments showed some evidence of local redistribution by turbidity currents, but sharp-based coarser-grained beds were entirely absent at Gardar Drift. We await shore-based X-radiography of the cores to confirm this general lack of evidence for currents.

Reworking of the nannofossil component was recognized throughout the sections and mean sedimentation rates are high: 51 m/Myr at Feni Drift and 58 m/Myr on Gardar Drift. No hiatuses were detected despite the excellent high resolution stratigraphic control. Deposition rates are higher on the drifts than in the region as a whole but no higher than at the sites that lacked the characteristic seismic features of drifts. We consider that slow, subtle redeposition of sediment is not confined to the drifts but is generally prevalent in the mid- to high latitude North Atlantic.

Closely-spaced 3.5 and 12 kHz profiles showed that individual sediment waves on both drifts are characteristically irregular in shape. Crest-to-trough variations in the sediments were slight but thickening and thinning of individual beds was recognized. We plan detailed shore-based grain-size and other analyses to look for local sedimentation differences. Although

there is little evidence on seismic profiles for sediment wave migration, systematic differences in accumulation rate curves from the crest versus trough holes at Gardar Drift appear best explained by wave migration at deeper Pliocene levels within the sequence.

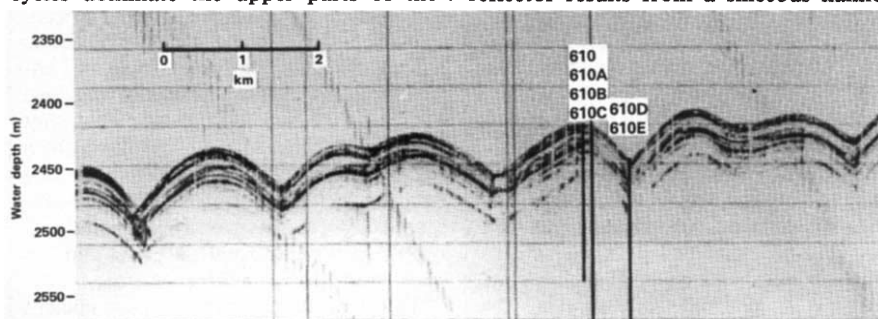
Deep drilling at Feni Drift penetrated an important regional seismic reflector at 0.75s sub-bottom (two-way travel time), which had been characterized as the base of the drift sequence, but more recently had been interpreted as a mid-drift reflector, marking the onset of modern deep circulation through Rockall Trough. The reflector results from a siliceous nanno-



Location of the sites drilled on Leg 94.

tude and up to a few kilometers in wavelength. Sites 610 and 611 were within wave fields on both drifts but at different levels in relation to their overall morphology. On Feni Ridge (Site 610) holes were drilled close to its sinuous axis at around 2,400 m water depth while at Gardar Drift (Site 611) drilling was on the lower south-eastern flank at about 3,200 m. Holes were drilled on a selected wave crest and offset holes were occupied in adjacent troughs.

Surprisingly, both drifts had fundamentally pelagic lithologies. As at the other Leg 94 sites, glacial/interglacial carbonate cycles dominate the upper parts of the



High-resolution seismic profile (3.5 kHz) of the sediment waves on Feni Drift, illustrating location of crest and trough drilling at site 610.

fossil chalk interval, upper lower Miocene (NN4) in age. No hiatus was present and we suspect from evidence of dissolution of the biogenic silica that a major oceanographic event took place at this time. Also within Feni Drift we sampled an upper Miocene chalk sequence, deposited at substantially reduced sedimentation rates and probably related to a major oceanographic event — in this case the Messinian isolation of the Mediterranean. Overall the upper halves of these two major drifts are comprised of sediments substantially younger than had been previously estimated.

Extension of the original palaeoclimate objectives at Site 608 to deep drilling allowed investigation of the origins of King's Trough, an anomalous bathymetric feature about 700 km north of the Azores. Many hypotheses for the origin of this intraplate tectonic complex of parallel ridges and basins have been put forward in the past fifteen years. The most recent hypothesis is based upon dredge hauls, rock cores and further geophysical data. It suggests an Eocene-Oligocene origin as an aseismic ridge built over a mantle hot spot that subsequently became rifted and subsided during the Miocene to form the deep troughs and flanking ridges of today.

Site 608 was drilled through the southern flank of King's Trough and sampled an upper Quaternary to upper middle Eocene sequence. Relatively fresh pillow lava basalts make up the basement; the inferred age (42 Myr) from the sediments in contact matches well the predicted age from magnetic anomaly identification. A major hiatus at 462 m sub-bottom separates an Eocene volcanoclastic sequence from upper Oligocene chalk conglomerates and nanofossil chinks, which display soft sediment deformation structures that are indicative of debris flow processes. Another interval with similar features and micro-faulting was found in the lower Miocene chinks. The remaining sequence is made up of nanofossil oozes and chinks, except for the glacial/interglacial cycles of marl and ooze that occur in the upper 76 m.

The detailed interpretation of the

tectonic events at King's Trough awaits re-examination of dredge haul and rock core data from within the complex itself. However, the timing of slope instability and volcanic activity in the drilled

sequence, along with the dating of the 11.6 Myr hiatus, appear to agree well with the postulated existence of an intraplate ridge in the late Oligocene and its subsidence in the Miocene. □

International ethological conference

The evolutionary basis of behaviour

from John R. Krebs and Robert M. May

In its early days, the science of ethology was devoted largely to the description of the behaviour patterns of animals. Today, however, the subject encompasses a wide range of disciplines, as indicated by the themes of the Plenary Sessions at the 18th International Ethological Conference*: parental investment; mating and mothering in farm animals; neurobiological approaches to hormonal mechanisms underlying behaviour; and the behavioural ecology of frogs, and of coral reef fishes. This ethological conference continued a trend set in the previous three biennial conferences, with an increasing number of papers treating aspects of the evolutionary basis of the behaviour of individuals or of social groups. Reflecting our own interests, our report concentrates on work of this kind.

How much should parents invest in offspring, and how should this investment be apportioned between sons and daughters? The answers to these questions are likely to vary with the environmental and biological setting of the species in question, and even to vary with an individual's age and status within a population, thus providing an arena where theoretical notions can be tested against field or laboratory investigations. Several such studies were reported at the meeting. Theoretical arguments¹ suggest that, for those animals producing offspring throughout their adult life, older individuals should devote relatively more metabolic and other resources to producing young than should younger individuals (essentially because the concomitant reduction in adult survival is less important toward the end of life). As discussed by Pianka and Parker¹, this pattern does seem to hold true for reptile and amphibian species. For birds, a study of California gulls by Pugesek² showed that the oldest gulls reared the most offspring: young parents (age 3–5 years) fledged on average 0.76 chicks per year; medium (7–9 year) aged parents reared 0.80 chicks; while older (12–18 year) couples fledged 1.50 chicks. For mammals, however, there has been little or no evidence in direct support of the predicted patterns. In a splendid review of the abundance of data that he and his collaborators have collected for red deer on the island of Rhum, Clutton-Brock (Cambridge University) suggested that, for

many mammalian species, older females may direct a larger proportion of available metabolic resources toward producing young, but that there may be a counter-vailing trend whereby the total amount of available resources declines with age. The outcome could easily be a biomodal curve, with relatively more young being produced by the youngest (most resources available) and oldest (highest proportion given to reproduction) animals, and relatively least by those of intermediate age. This, indeed, appears to be the pattern found among Clutton-Brock's deer.

Whatever the level of parental investment, theory suggests that — other things being equal — the investment should be partitioned equally between sons and daughters. This result, established by Fisher³ and elegantly generalized by MacArthur⁴, arises because in a population where sons were rare a female who produced an excess of sons would have an above-genetic input into the next generation (and conversely if daughters were rare); hence the evolutionarily stable state is one of equal investment. For most mammals, sons and daughters 'cost' much the same to produce and rear, so that 'equal investment' translates into a 50:50 sex ratio. Other things are, however, not always equal. Thus, as we have previously discussed⁵, a haplodiploid reproductive scheme can introduce many complexities into sex ratio determination; further empirical and theoretical work on this subject was reported at the conference, and will be discussed in a forthcoming *News and Views* article.

Another kind of exception to the Fisher-MacArthur expectation arises in promiscuously mating species, where the variability in the reproductive success of males clearly can be significantly greater than that of females, with a handful of dominant males sometimes being responsible for most of the copulations. Trivers and Willard⁶ have observed that, in these circumstances, if some females are able (by virtue of higher rank in a dominance hierarchy, or because times are good, or for some other reasons) to produce sons of above-average robustness — which consequently are likely to have above-average reproductive success — then these females would do well

*The Eighteenth International Ethological Conference was held in Brisbane, Australia, on 29 August–6 September 1983.

The scientific party aboard D/V *Glomar Challenger* for Leg 94 of the Deep Sea Drilling Project, International Phase of Ocean Drilling, consisted of:

Robert B. Kidd (Institute of Oceanographic Sciences, Wormley, Godalming, Surrey, United Kingdom).

William F. Ruddiman (Lamont-Doherty Geological Observatory, Palisades, New York)

James F. Dolan (University of California at Santa Cruz, Santa Cruz, California)

Margaret R. Eggers (University of South Carolina, Columbia, South Carolina)

Philip R. Hill (Geological Survey of Canada, Dartmouth, Nova Scotia, Canada)

Lloyd D. Keigwin, Jr. (Woods Hole Oceanographic Institution, Woods Hole, Massachusetts)

Margie Mitchell (Scripps Institution of Oceanography, La Jolla, California)

Isabelle Philipps (Universite de Bordeaux I, Talence, France)

Frank Robinson (Lamont-Doherty Geological Observatory, Palisades, New York)

Sasson Salehpour (University of Rhode Island, Kingston, Rhode Island)

Toshiaki Takayama (Kanazawa University, Kanazawa, Japan)

Ellen Thomas (Deep Sea Drilling Project, La Jolla, California)

Gerhard Unsold (Geologisch-Palaeontologisches Institut und Museum der Universität Kiel, Kiel, Federal Republic of Germany)

Philip P.E. Weaver (Institute of Oceanographic Sciences, Wormley, Godalming, Surrey, United Kingdom)

to produce more sons than daughters. Applied to Clutton-Brock's red deer, these ideas say, first, that females may in general tend to exhibit a male bias in their offspring in favourable years, and a female bias in bad years; and second, that higher ranking females (which are usually in better condition, have higher reproductive success, and may be inferred to be producing more robust offspring) should exhibit a bias toward males (and the lower ranking females a bias toward females) at all times. The data in support of the first expectation are equivocal, but the second expectation is confirmed in a statistically significant way: higher ranking female red deer on Rhum produce a higher ratio of sons to daughters than do lower ranking females (*Nature*, in the press).

The study of mate choice is another rich area for ethological investigation⁷, with broad implications both for the genetic structuring and divergence of populations and for the much discussed and still controversial^{8,9} theory of sexual selection¹⁰. Several papers at the conference were concerned with analysing the criteria of mate choice and exploring its relationship to the evolution of sexual adornments.

In an intriguing study of captive zebra finches (*Poephila guttata*), monogamous Australian estrildines, Burley (University of Illinois) demonstrated that both sexes can be influenced in their choice of partners by putting coloured plastic rings on the tarsus ('colour-ringing' is a standard technique used by ornithologists to allow individual recognition of birds in the field — Burley's results provide a sobering *caveat* for these people). By measuring the time spent in front of members of the opposite sex in a choice chamber, Burley could show that test females prefer to be near red-ringed males over those with orange or green rings, while males go for black in preference to blue or orange. Control trials showed that the preferences hold only for members of the opposite sex. It is quite puzzling why a total artifact such as a colour ring should affect mate choice. Perhaps the rings happen to trigger the normal sexual recognition mechanism, just as in the classical ethological demonstrations that animals can be induced to court quite un-lifelike dummies (even in preference to the real thing) as long as the dummy exhibits certain key stimuli. The female preference for red rings, for example, may be related to the fact that the breeding male has a bright red bill.

Whatever the mechanism underlying the effect, Burley was able to use the fact that colour rings influence attractiveness as a tool to look at the consequences of mate preferences. She put 24 unringed males and 24 ringed females (8 with each of the 3 colours used in the initial preference trials) in an aviary, allowed them to pair up, and measured the reproductive success (number of young reared to independence) of each pair over a long period. The most attractive females (black) had the highest

success and the least attractive (blue) the lowest. Why? The answer is not yet fully worked out, but the current suggestion is that attractive females get more parental help from their husbands in feeding the young and, because they are less stressed, live through more breeding cycles. In this artificial experimental set-up it is unlikely that males derive any benefit from choosing attractive females (since the female's extra production of offspring is dependent on male effort) but in natural situations choice by either sex must presumably confer an advantage. Exactly what this advantage might be is a hotly debated topic.

According to the theory of intersexual selection, certain display characteristics may be preferred purely because their bearers pass on the attractive traits to their offspring, giving them a mating advantage. By choosing partners with the attractive trait, an individual increases its offspring's success and hence passes on its genes more effectively. This idea, first developed by Fisher³ fifty years ago, has a certain stunning inevitability about it. Once a preference gets started (several suggestions for ways in which this could happen are current in the literature) it develops its own positive feedback to cause the evolutionary elaboration of preferred display traits. Side-stepping the mathematical minefield of argument about whether, and under what conditions, Fisher's neat idea will work, one can ask whether there is any evidence that the extravagant displays traditionally associated with sexual selection really do attract sexual partners. An apparently convincing case (one of the few so far documented) was reported by Borgia (University of Maryland).

The closely related bowerbirds and birds of paradise have (to quote from the extraordinarily beautiful book by Forshaw and Cooper¹¹) "... held for more than four centuries an indisputed reputation as the most fascinating of all birds. It is only when confronted with a living bird that an observer can fully appreciate the extreme beauty and exquisite adornments". While male birds of paradise carry their adornments on their bodies as plumage, the bowerbirds construct sexual adornments in their environment in the form of intricate bowers of twigs, leaves and coloured objects. As Borgia showed, this produces an ideal system for quantifying and manipulating adornments. In this study of the Australian Satin Bowerbird (*Ptilonorhynchus violaceus*) he used automatic cameras to record continually the mating activities at more than 20 bowers. The male's bower consists of a platform of interwoven twigs with an avenue 30cm long by 10cm wide of two parallel walls of twigs, the tips of which are arranged to curve in at the top. The walls are coated by the bird with chewed vegetation and the platform, especially on the north side of the avenue, is decorated with blue feathers or flowers, yellow leaves,

snail shells, cicada skins and other items. Blue is the favoured colour for decorations (perhaps mirroring the male's own bluish-black plumage) and when available the flotsam of camp and picnic sites is used: blue drinking straws, toothbrushes and even the odd tube of Foster's lager.

During the breeding season the male attends and decorates his bower regularly and females visit it for mating. Correlation analysis and manipulations of bowers showed that variation in mating success among males (0–33 copulations per season in Borgia's area) is dependent on the number of blue feathers and snail shells in the bower. More elaborate bowers both attract more female visits and are more likely to lead to successful mating once visited. Whether or not the female's preference for bowers is maintained by Fisherian sexual selection remains an open question. An alternative possibility is that the bower is a direct indicator of overall male quality (which given some genetic variation might be passed on to the female's offspring). In Borgia's population, males accumulate decorations in part by stealing from other bowers, so that a male's bower is a reflection of his ability to steal from others and prevent others stealing from him.

In yet other studies of mate choice, Jouventin and Weimerskirch (Laboratoire Zoologie, Montpellier) showed — by removing the yellow feather crests on a control group — that the head ornaments on rockhopper and macaroni penguins play an important role in initial pair formation. And, in contrast to some earlier reports, Bradbury's (University of California at San Diego) field studies of Californian populations of sage grouse showed that quantitative measures of the vigour and frequency of display by males were good predictors of female choice. Taken together, these several studies of what might be called aesthetic aspects of mate choice not only advance our understanding of sexual selection, but also raise fascinating questions about the evolutionary origins of our own aesthetic sensibilities. □

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Molecular biology

Exons — present from the beginning?

from Colin Blake

SINCE introns and exons were discovered six years ago, the split organization of eukaryotic genes has been seen as a mechanism for enhancing the rate of evolutionary change. First, Gilbert¹ put forward the important hypothesis that exons correspond to protein functions which can be reshuffled by recombinations within introns to produce new kinds of proteins from parts of existing ones. This hypothesis was extended by Blake² who suggested that if exons also correspond to folded protein structures, domains or smaller units, the problem of folding the new protein would be made easier. Gō³ developed this suggestion into an analytical tool for testing the correlation between gene structure and protein structure, by using the diagonal plot (now known as the Gō plot), and successfully predicted the location of the third intron in the leghaemoglobin gene. Doubts about the correctness of these original hypotheses arose, however, as other and more recent gene structures can only be fitted into them with considerable difficulty. The publication of three new papers on the gene structure of enzymes now prompts a reassessment of the origin and significance of the split-gene structure.

Campbell and Porter⁴ have reported that in complement factor B the coding region for the serine proteinase domain is divided into eight exons with each of the important catalytic and binding residues located on different exons. Gō⁵ has analysed the chicken lysozyme gene using the diagonal plot and in suggesting the location of another intron has pointed out that its presence would separate the major catalytic and binding residues located on different exons. Craik *et al.*⁶ have noted precisely the same effect in the carboxypeptidase gene.

Campbell and Porter suggest that the presence on a separate exon of the amino acid that confers substrate specificity on the serine proteinases could make it easier to interchange the specificity-conferring sequences between the many different enzymes of this group. This may be true, but the classic studies of pancreatic serine proteinases have suggested that the specificities of chymotrypsin, trypsin and elastase are determined by one to three amino acid substitutions which appear to be the result of point mutation. Although it is not possible to generalize for all enzymes, it seems more reasonable to consider the catalytic activity of lysozyme, carboxypeptidase and the serine proteinases as a single function, rather than to break it down into a catalytic function and a series

of binding and specificity functions. In these enzymes, the binding of the polymeric substrate is determined by the overall conformation of main chain and side chains, and it seems unrealistic to think, for example, of subsite B of lysozyme as an independent entity capable of carrying its particular sugar-binding function into another protein. The reality is surely that the catalytic function of these enzymes is encoded by a number of exons, not one as proposed by Gilbert.

Study of other proteins leads to the same conclusion: the three specific binding functions in serum albumin appear to be each

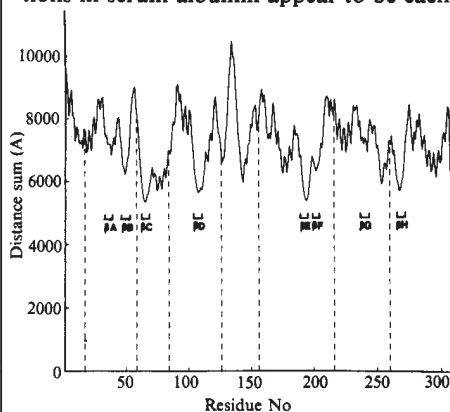


Fig. 1 Plot of the inter-residue distances for each residue of bovine carboxypeptidase. This is equivalent to a 'Gō plot' in which the 'valleys' along which the splice junctions are claimed to run correspond to minima in the distance sum. The splice junctions in the rat carboxypeptidase gene⁶ are marked by broken lines. The locations of the eight β -strands in the central β -sheet of the carboxypeptidase molecule are shown. I am grateful to Simon Evans for this diagram.

encoded by four exons⁷; the serine proteinase binding function in ovomucoid by two exons⁸; and even the haem binding function of haemoglobin, encoded by a single exon in many animal haemoglobins, is carried by two exons in the plant leghaemoglobin⁹. Although there are functions encoded by single exons, it is more common for functions to be encoded by several exons. As a corollary protein domains are usually encoded by more than one exon.

It is also necessary to point out that the Gō analysis of the correlation of exons with protein structure does not have general applicability. Despite its remarkable success in predicting the location of the additional intron in leghaemoglobin, the method is too correlated with certain types of structure to be useful in identifying protein 'modules' that appear to be

encoded by separate exons in haemoglobin³ and lysozyme⁵. Figure 1 shows the Gō plot for bovine carboxypeptidase drawn so that the inter-residue distances can be quantified. It shows that the minima in the plot do not in general coincide with splice junctions in the rat carboxypeptidase gene, but rather correspond to locations of the β -strands in the molecule. This is easily understood because in the α/β class of proteins, of which carboxypeptidase is a member, the β -sheet runs close to the centre of the molecule where the inter-residue distances are a minimum. Gō³ acknowledged this effect in haemoglobin, where it is minimized by the two-layer structure in which chain segments do not get close to the molecular centre, but it clearly dominates the plot in the common three- or four-layered α/β structures.

Thus none of the current ideas on the relation of coding sequences to protein function and structure seems fully correct. Rather than simply discarding them, however, it may be possible to place them in a more comprehensive context which takes into account a range of other studies. The most crucial point comes from the determination and analysis of the fine structures of the collagen and α -fetoprotein genes. In the triple-helical region of the 52-exon α_2 -procollagen gene from chicken, the exons are either 54 base pairs (bp) in length or are simply related to an exon of this size¹⁰. The direct, or derived 54-bp exons are not only of the correct size to code for the sixfold repeat of the fundamental Gly-X-Y sequence of the collagen helix, but are also in register with it. This seems compelling evidence for the evolution of the 1,000-residue collagen molecule by repeated tandem duplication of a single ancestral 54-bp exon.

Similarly, there is clear evidence that the 14 exons in the coding region of α -fetoprotein are also derived from a single ancestral exon⁷, apparently 27 bp long¹¹. An analysis of the number of exons in a representative sample of proteins (Fig. 2) indicates that there is a good overall correlation between exon number and chain length, with two to three exons per 100 residues. This is consistent with the view that the proteins were assembled from several smaller exon-encoded units. A histogram of exon size for the same 20 proteins (Fig. 3) indicates a relatively sharp distribution about a mean length of 40–45 residues per exon. It is interesting to note that Wetlauffer²² has concluded that the minimum size of peptides that can assume a stable folded structure is in the range 20–40 residues. A preliminary examination of the few proteins of known three-dimensional structure and gene structure suggests that exon transcripts frequently, but not always, correspond to folded segments of supersecondary structure.

If we now make the general assumption that exons are relics of the original 'assembly' of proteins what implications

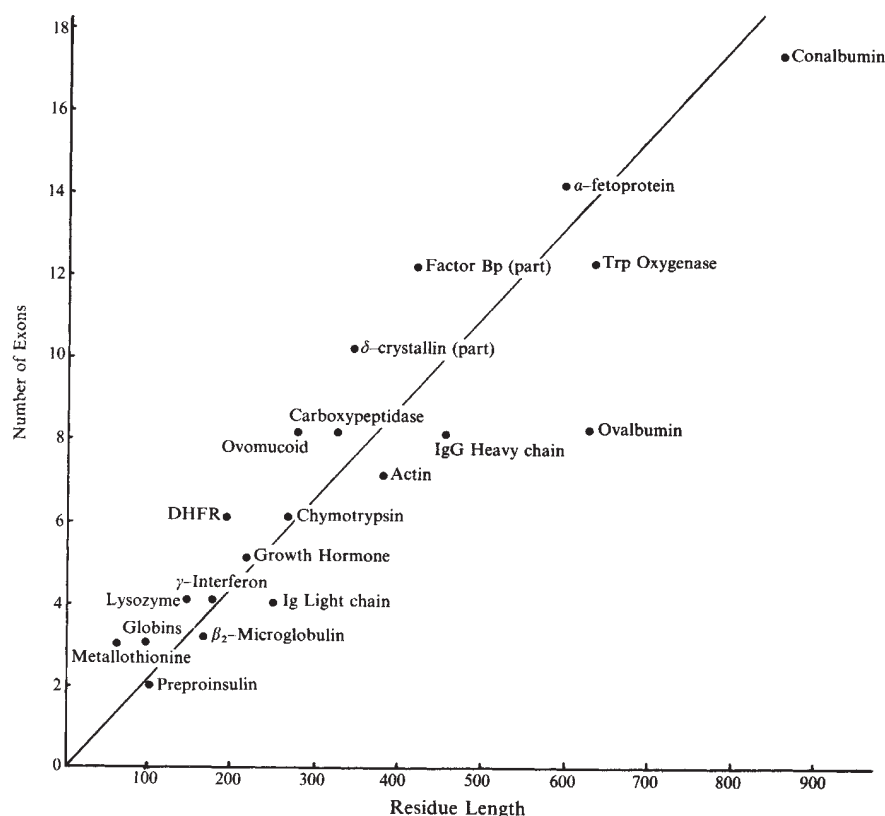


Fig. 2 Plot of the number of exons against chain length for 20 different proteins. The full line represents one exon per 45 amino acid residues (see Fig. 3).

will it have? The most obvious consequence is that the ancestors of both the eukaryotes and the prokaryotes would have to have had a 'split gene' organization. Although it was initially assumed that the precursor gene structure was more like that of the prokaryotes, and therefore without introns, Doolittle¹² argues persuasively that the idea that introns were introduced in eukaryotes to increase their evolutionary potential imputes purpose, and is thus non-Darwinian. He suggests instead that the presence of introns in early cells would not only have evolutionary significance but also be able to compensate for their potentially unfaithful transcription and translation systems. Subsequently, introns were lost in the prokaryotes in a 'streamlining' process that traded efficiency for evolutionary potential, but retained in the eukaryotes which followed the opposite path. Unexpected support for this view has come from the discovery of introns in the ribosomal RNA genes of archaeobacteria¹³, which may represent a third cell type that branched from the common ancestor earlier than the prokaryotes and eukaryotes. Other evidence that may be seen to support an early 'split gene' organization comes from considerations of the stability and direction of change of gene mosaics. Although the positions of introns are relatively stable, for example the precise splice junctions in haemoglobin/myoglobin have been maintained for more than 800 Myr (ref. 14), they are

not totally stable and a number of examples of genes with variable numbers of introns are known. Where it is possible to define the process giving rise to different numbers of introns, for example in actin¹⁵, collagen¹⁶ and insulin¹⁷, the process seems to involve the deletion of introns rather than their introduction. It is probably too early to be certain but if deletion of introns is the dominant event, and a single process is assumed, then ancestral genes should have more, rather than fewer, introns.

A further piece of evidence comes from hints that the splicing process itself has been subject to evolutionary change. The most surprising discovery is that the intron in the rRNA of *Tetrahymena* is excised by the intron acting as its own cleavage-enzyme¹⁸. This requires the intron to take up a specific three-dimensional structure at least in the vicinity

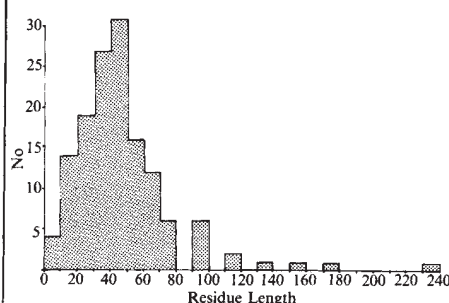


Fig. 3 Histogram of exon sizes in amino acid residues for the 20 proteins plotted in Fig. 2.

of the splice junction. The introns in mitochondrial RNA also seem to form complex secondary structures¹⁹, but here enzymes, maturases encoded by the introns, carry out the splicing. The removal of introns from nuclear pre-mRNA may require another and more general splicing system, less dependent on intron structure²⁰. It seems probable that the different splicing mechanisms represent an evolutionary process for adding and refining regulatory control to the splicing process. If this is correct, then the self-removal of the rRNA intron is probably the primitive form, depending only on the intron sequence, and possibly representative of the early splicing process.

The accumulation of new evidence makes it possible to attempt to produce a comprehensive view of the origin and role of exons in protein evolution. (I have become aware that Gō⁵ and Ohno²¹ have also argued along these lines.) It begins with early coding units that expressed small oligopeptides of, say, 20–40 residues. Some of these may have been potential supersecondary structures, such as the $\alpha\alpha$, $\beta\beta$ and $\beta\alpha$ structures that make up contemporary proteins. Small folded structures of this kind could be readily assembled to form larger peptides representing early protein molecules (this could be an explanation of the observation by Craik *et al.*²³ that splice junctions tend to map at protein surfaces), but it is not necessary to think in terms of rigid correlations because the structural potential of any small unit could be modified by its environment in the protein. The flanking noncoding sequences would be such as to allow their self-excision. Tandem duplications, and possibly shuffling, of these small coding sequences together with their flanking segments could lead to early transcription units with exon-intron mosaics that coded for stable protein molecules made up from individual supersecondary, and other, structures. A large number of different products could be derived from a small number of ancestral exons if the order in which exon duplication, exon shuffling, intron deletion and mutations at splice junctions changed. With the establishment of the exon-intron mosaic, the evolutionary mechanisms outlined by Gilbert could come into play to build larger multifunctional multidomain protein molecules, with the difference that protein functions would usually be encoded by a block of exons. Since prokaryotes contain complex proteins, this aspect of the evolution of many proteins would have been largely completed in the ancestors to the prokaryotes and eukaryotes, or in the prokaryotes before the loss of their introns (which would effectively prevent any further evolution in this direction).

This proposal for the origin of exons attempts to remove Doolittle's objection to Gilbert's hypothesis, and to modify the detail of the latter without losing its substance. The critical point is, of course,

whether early cells had split-genes; and this question might be finally settled if exon-dependent evolution can be established for eukaryotic proteins which have defined homologues in prokaryotes. □

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Immunology

The I-J paradox remains unsolved

from Alan Munro

THE I region of the mouse major histocompatibility complex (H-2) codes for cell surface glycoproteins referred to as class II molecules but previously known as I-region associated or Ia antigens. The class II molecules are heterodimers of two transmembrane glycoproteins, a 32,000 molecular weight (MW) α chain and a 28,000 β chain. In the mouse they are referred to as I-A, containing A_α and A_β chains and I-E containing E_α and E_β chains¹. These molecules are expressed on the surface of B lymphocytes, macrophages and other antigen-presenting cells and are important in the interaction of T cells with antigens². They were originally detected serologically using allo-antisera raised by immunizing one mouse strain with cells from a different strain. From the patterns of reaction of numerous allo-antisera on cells from different mouse strains, particularly those carrying H-2 recombinations, it was shown that genes in the I region controlled the antigenic determinants on I-A and I-E molecules. The simplest interpretation is that the genes for the A_α , A_β , E_α and E_β chains lie within the I region and almost a year ago Hood and his colleagues presented a molecular map of the mouse I-region which showed this to be the case. They found a single gene for three chains and possibly two for the E_β , though $E_\beta 2$ may be a pseudo gene. The order of the genes in Balb/c mice is 5' — A_β — A_α — E_β — E_α — 3'.

The I-region contains genes which affect the antigenic determinants on other molecules apart from I-A and I-E and of these I-J has attracted most attention. Antisera to I-J will react with suppressor T cells and antigen-specific T-cell suppressor factors, and there are now monoclonal antisera with this specificity, including antibodies derived from reciprocal immunization between the two mouse strains B10.A(3R) and B10.A(5R) (see ref.4). These two

strains were derived as an intra-MHC crossover between the same parental strains C57BL/10 and B10.A and were thought to be identical until the discovery of I-J. The simplest interpretation is that the crossover carried by B10.A(3R) and B10.A(5R) occurred at different places and that the genetic information between the crossover (points) codes for I-J molecule. Unfortunately, this time the simplest interpretation appears to be incorrect.

Using the restriction enzyme site polymorphisms associated with the H-2 types of the two parental strains, it has been possible to pinpoint the crossovers in B10.A(3R) and B10.A(5R) to a 3.4 kb region that lies within the transcribed sequence of the E_β gene and includes part of one of the E_β exons³. There are two types of models to explain this apparent paradox between immunogenetics and molecular genetics⁵; either the I-J gene is all or part of the E_β gene or the I-J gene is situated elsewhere and the differences in H-2 associated with B10.A(3R) and B10.A(5R) mice indirectly influence the antigenic determinants on I-J^{6,7}.

Two recent papers provide further information to help one towards a possible explanation^{7,8}. Several groups have been able to isolate T-cell hybridomas which secrete antigen-specific suppressor factors carrying I-J and these have now been used to search for a messenger RNA transcript of the putative I-J region. Poly(A) tailed RNA from 13 hybridomas has been subjected to northern blot analysis using two DNA probes that span the whole of the E_β gene including the differences between B10.A(3R) and B10.A(5R) (see ref.7). In no case was any messenger RNA found that hybridized with these DNA probes in spite of the fact that several of the hybridomas produce suppressor factor which reacted with B10.A(3R) and B10.A(5R) or the converse antisera. In these experiments it was

estimated that the level of detection of mRNA was 3–4 copies per cell. Further the DNA from four of the hybridomas was compared to liver DNA obtained from mice with the appropriate H-2 haplotype and there was no apparent rearrangement of the hybridoma DNA in this region⁷.

If I-J is coded for by the region defined by the crossover in B10.A(3R) and B10.A(5R), then it must have an unusual messenger RNA though it is unlikely that it simply lacks poly(A) tails, as poly(A)-tailed RNA from suppressor T-cell hybridomas has been translated to produce suppressor factor-bearing I-J^{9,10}.

The alternative approach to solve this dilemma is to characterize the I-J molecules. This has been more successful with the I-J positive suppressor factors than with the cell surface I-J and two forms of I-J bearing suppressor factor have been identified¹¹. One consists of a single chain of about 25,000 MW and the other is a 60–70,000 MW dimer of two chains, one of which binds the antigen and the other carries I-J determinants. Interestingly, both types of factor react with a B10.A(3R) anti B10.A(5R) antiserum¹².

In a series of experiments analysing suppression of the response to lactic acid dehydrogenase (LDH), Klein and his co-workers have discovered a T-cell hybridoma with unusual properties^{8,13}. This hybridoma secretes two antigen-specific suppressor factors both of which contain two chains. In each case the chain which binds antigen is associated with a chain carrying I-region determinants. With one factor (TsF-E) the second chain reacts with anti I-J (defined by B10.A(3R) anti B10.A(5R)) and anti E_β monoclonal antibodies and with the second factor (TsF-A) the chain carrying I-region determinants reacts with monoclonals to A_β , a crossreacting I-J determinant and T cell I-A determinants. The latter determinants are defined by monoclonal antibodies which react with suppressor factors but by immunogenetics map to I-A (see ref.14). These workers also show that the two different suppressor factors react with different cells; TsF-A suppresses helper T cells responding to LDH in the context of A_β (that is, A_β restricted helper T cells) and TsF-E suppresses helper T cells responding to LDH in the context of E_β ⁸. They argue that in order to be active the factor has to interact with its target via an antigen bridge and at the same time via a receptor for I-region determinants on the factor. Since the same polypeptide chain apparently carries E_β and I-J determinants it is possible that I-J is a modified determinant of E_β and coded for by the E_β gene. A similar argument would apply to T cell I-A determinants and A_β . However, this cannot explain the failure to detect E_β transcripts in the other I-J positive hybridomas mentioned above.

It is possible that the apparent 'dual recognition' of the suppressor factor by

helper T cells may provide the answer. There is ample evidence that while interacting with antigen, helper T cells in some way recognize class II molecules expressed on the surface of antigen-presenting cells. If the same receptors involved in this interaction recognize the suppressor factor, then the factor may carry a determinant that crossreacts with a class II molecule but is coded for by genes outside H-2. Regulatory interactions between cells of the immune system would have had to select for suppressor cells with receptors which combine with antigen and in addition carry determinants which crossreact with the appropriate class II molecules. Different H-2 genes would lead to different cross-reacting determinants on the antigen receptors on suppressor T cells and on antigen-specific suppressor factors. This type of argument is not new and has been used to explain the presence of immunoglobulin heavy chain V region idiotype determinants on T cells and on T cell antigen specific factors even though it is impossible to detect the expression of V_H genes in T

cells¹⁵. In both cases one would expect the apparent phenotype of the T cell as far as I-J and idiotype are concerned to depend on the genotype of the environment that the T cell developed in. One feels that solution of this paradox will only be found once more is known about the genes that code for the antigen receptor on T cells. □

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Immunology

HLA class II antigens and monoclonal antibodies

from Stephen Shaw and Andrew McMichael

At a recent workshop* in Edinburgh investigators from forty different laboratories grappled with the idiosyncratic properties of more than 100 monoclonal antibodies raised against HLA Class II antigens. These glycoproteins, also known as Ia antigens, are of great interest because of their importance in transplantation reactions and their association with susceptibility to a variety of diseases. These properties are probably related to their key role in the presentation of antigens to T lymphocytes.

It was evident that participants at the meeting agreed on a number of important points. There are three families of closely related Ia molecules encoded by the HLA D region: one designated DR, one designated SB and one variously described as DC, MB or DS (called DC hereafter). It is known that DR and DC are respectively equivalent to the I-E and I-A antigens of the mouse H2 complex^{1,2} (see also *News & Views* **304**, 214; 1983) and recently E. Long (University of Geneva) found preferential cross hybridization between an SB β cDNA probe and the E β 2 (?pseudo) gene of the mouse H2 complex, suggesting that SB too might have a murine analogue. There are however, further layers of complexity in the HLA system. Each Ia molecule is an heterodimer of two polypeptide chains — α

and β — and is encoded by two genes in the D region. DNA sequencing and Southern blotting indicate that for the DR, DC and SB families, respectively, there are at least three, two and two β chain genes per haplotype. Similarly, there are at least two DC α chain genes. DR, DC and SB β -chain genes and the DC α genes are known to be polymorphic in the population.

With this knowledge of the genetic background, the principle task of the workshop was to distinguish serologically individual gene products, with the long term aim of identifying their function. It was hoped that the large number of monoclonal antibodies raised against HLA Class II antigens would be sufficient; but the workshop showed that most of the available antibodies cannot be relied upon to make such distinctions. This is because most reagents show complex patterns of cross reactivity between alleles and between different families of molecules. Two features of the system predispose to detection of shared determinants. One is the extreme homology of different Ia antigens: 95 per cent nucleotide homology is seen between β -chain alleles and 75 per cent homology between β genes of the SB, DR and DC families (C. de Preval, University of Geneva). Second, the use of xenobodies allows recognition of species specific determinants. In this context it was noteworthy that some of the more interesting allotype specific reagents were made from rat rather than the usual Balb/c

mouse spleen cells (S. Radka, University of Philadelphia).

The cross reactions observed make very careful evaluation of any given antibody essential. For example, one antibody that detects DR on all haplotypes, detects the DC molecule of the DR7 haplotype only². Another recognises the SB2 and SB3 allelic products but also DRS of the DR series³. Furthermore, the observed antigen specificity of an antibody can vary depending on the 'readout'; crossreactivity is less evident with immunoprecipitation (which typically depends on fairly high affinity binding) than with assays of binding. In short, extreme caution is warranted.

Some hope was offered by the description of a small but increasing band of monoclonal antibodies that recognise epitopes present only on some members of the D family, 'polymorphic monoclonals', (J. Johnson, University of Munich; S. Radka, University of Philadelphia). An example of what could be revealed came from S. Fuggle and D.Y. Mason (University of Oxford) who identified unique patterns of binding to lymph node frozen sections of two of the 101 monoclonal antibodies. The mantle zone of the lymphoid follicle stained intensely whilst cells in the germinal centre were only weakly positive. All other antibodies stained germinal centres strongly. Immunochemical analysis of the products detected by these antibodies showed them to have the general structure of Ia and mapping on the HLA-loss mutant cell lines developed by R. deMars (University of Wisconsin, Madison) documented control by genes of the DR-subregion. However, they were shown to discriminate between DR molecules: they bind to products of only some HLA haplotypes in the population, typically those encoding DR3, DR4 or DR7 (H. Festenstein, University of London; J. Johnson, University of Munich; W. Liebold, University of Nurnberg); and one of the two antibodies, which has been studied in detail by D. Capra and R. Giles (University of Texas), immunoprecipitated only two of the three DR β chains. The indication that there could be differential expression of particular Ia antigen subpopulations was supported by the findings of R. Winchester (State University of New York) and P. Stastny (University of Texas, Dallas) who found preferential expression of DC molecules on B cells relative to macrophages and the finding that some chronic lymphatic leukaemia cells apparently fail to express DC (K. Guy, University of Edinburgh and D. Charron, University of Paris).

Biochemical studies appeared to resolve some controversial issues, but again added complexity. Progress was made in understanding the relationship between the serologically defined 'supratypic' specificities and these families of molecules. The MB1, MB2 and MB3 specificities are present on alleles of the DC family of molecules (R. Tosi, University of Rome; G. Corte, Uni-

*The First International Workshop on Monoclonal Antibodies to Human Major Histocompatibility Complex Class II Antigens was held on August 29-September 2, 1983. The meeting was sponsored by the British Society for Immunology and convened by Michael Steel, MRC Cytogenetics Unit, Edinburgh.

versity of Genova, J. Silver, University of Michigan; D. Capra, University of Texas, Dallas). In contrast the *MT2* and *MT3* specificities are present on at least a subset of molecules of the *DR* family (R. Tosi, University of Rome; D. Capra). On another front, biochemical techniques have begun to uncover a great deal of heterogeneity among haplotypes (and molecules) which are serologically indistinguishable but which have been inferred to differ on the basis of functional recognition by T cells. *DR4* haplotypes were elegantly dissected by P. Antonelli (Fred Hutchinson Cancer Center, Seattle) and C. Navarette, University of London) into distinctive combinations of six different *DR4* β variants and 3 different *DC β* variants. These haplotypes correlated with the *Dw* type determined by mixed lymphocyte reaction.

Potentially the most exciting result reported at the workshop was that of C. de Preval (University of Geneva) who had introduced a single pair of HLA *DR α* and *β* genes into mouse L cells and obtained expression of Ia antigen on the cell surface⁴. When the expressed antigens were precipitated by monoclonal antibodies (R. Acolla, Ludwig Institute, Lausanne) two subsets of antigens were distinguished by sequential precipitation and peptide mapping. The differences seemed too great to be accounted for by glycosylation differences alone and the observation raises the possibility of alternative splicing of mRNA. If this result is not a peculiarity of these transfected L cells, it would add yet another layer of complexity to the Ia antigen system.

Finally, what of function? The role of Ia antigen presentation and initiation of mixed lymphocyte responses is of enormous interest. However, given the complexity of the binding patterns of the monoclonal antibodies currently available it is very difficult to evaluate their properties when added to lymphocyte cultures *in vitro*. They clearly have effects, however, and both inhibition and enhancement of functional responses were observed (A. Moretta, Ludwig Institute; W. Liebold, University of Nurnberg; M. Taylor, University of Manchester; F. Koning, University of Leiden). A clearer picture emerges when the effect of monoclonal antibodies on cloned T cell recognition of antigen was studied (G. Ball, University of Texas and C. de Freitas, University of Philadelphia). Here it was possible to correlate the restriction specificity with the product identified by the monoclonal antibody allowing a stronger link

between a (family of) molecules and a particular T cell response. Much further work is needed in this area to determine the relative importance of particular subsets of human Ia antigens in initiating normal polyclonal immune responses.

In conclusion, it is clear that monoclonal antibodies are capable of dissecting the complicated HLA *D* system. Each reagent must be carefully evaluated for its reactivity in different HLA haplotypes. This is most easily done for those that seem locus specific or detect a polymorphic specificity and these reagents should be most valuable in looking for differential expression and function. There is still a place however for truly monomorphic reagents and those

which seem to detect new Ia antigens^{5,6} (R. Ziegler, University of Tübingen); only these can identify the full range of products expressed. It remains to be understood why there are striking differences between the man and the mouse in the number of defined human Ia genes, and their expression on cells other than B cells/macrophages. Do they reflect a fundamental difference between man and mouse in the way these antigens are currently used or has such divergence emerged in a way that is functionally neutral? □

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Immunology

Mitochondria and the major histocompatibility complex

from Peter Goodfellow

Two new papers appearing in *Nature*^{1,2} add those with an interest in mitochondria to the ever-growing list of scientists required to understand something of the major histocompatibility complex (MHC). Not so many years ago the MHC was commonly thought to be too complex to be comprehensible to all but a few dedicated serologists. But, since then, a combination of classical, cellular, and biochemical genetics has revealed a basic simplicity in the structure of the MHC.

The region contains three major classes of genes: Class I genes coding for 45,000 (45K) molecule weight glycopeptides which associate with β_2 -microglobulin (which is not encoded by the MHC); class II genes coding for associated α and β glycopolypeptides of approximately 32K and 28K; and class III genes which code for components of the complement system. Class I and II products are directly involved in immune responses. The class I genes are responsible for graft rejection and the formation of T-cell recognition structures and class II genes produce recognition structures used in lymphocyte cellular interactions.

Recent molecular genetic studies have shown, however, that this apparent simplicity is, in part, illusory: although the products of less than ten class I genes have been described, 30 to 40 class I genes exist in the mouse³ and human genome⁴. Similarly, several class II genes have been described⁵.

Many roles have been postulated for this plethora of genes: over 10 years ago, for example, it was suggested that tissue specific class I gene expression might regulate normal development⁶. In addition, there are many phenotypic traits associated with the MHC which require controlling genes; these traits range from disease susceptibilities⁷ to variations in ligand

binding⁸ and septal formation in the vagina⁹. What was not predicted and not expected was that some class I genes of the MHC might be regulated by mitochondrial genes.

In a series of experiments undeterred by prevailing dogmas Kirsten Fischer Lindahl and her colleagues demonstrated that an antigen, Mta (maternally transmitted antigen), is inherited maternally¹⁰⁻¹². The Mta⁺ phenotype is common to most, but not all, strains of inbred mice and is defined either by T-cell killing or by transplantation rejection. In addition to maternal inheritance Mta is unique because unlike other weak transplantation antigens it is recognised by T cells in the absence of associated class I products. Embryo transfer and new-born fostering experiments do not modify the maternal inheritance pattern of Mta, strongly suggesting transfer of the responsible controlling element through the egg cytoplasm. As Fischer Lindahl suggests, the obvious candidate for the controlling element is the DNA of mitochondria. Support for the view comes from two observations. First, the mitochondrial DNA from Mta⁺ and Mta⁻ strains of mice differ in sequence^{13,14}. Second, as shown by R. Smith, M. Huston, R. Jenkins, D. Huston and R. Rich of Baylor College of Medicine, Houston, in this issue of *Nature* (p.599) Mta⁺ antigen expression in somatic cell hybrids produced between Mta⁺ and Mta⁻ cells requires the presence of Rhodamine 6G sensitive structures derived from the Mta⁺ cells. As mitochondria are the only organelles known to be sensitive to this drug these results give strong support to the view that mitochondrial DNA controls Mta⁺ expression. The responsible mitochondrial gene has been named *mtf* (maternally transmitted factor).

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Although inheritance of control of antigen expression is cytoplasmic the structural gene for the Mta antigen appears to be a class I MHC gene. The genetic evidence for this contention has just been presented in *Nature* by K. Fischer-Lindahl, B. Hausman and V. Chapman (see p.383). The critical observation is that two Mta⁻ strains of mice can complement each other to produce Mta⁺ offspring. One of the Mta⁻ strains carries the cytoplasmic defect previously defined, the second Mta⁻ strain carries a defect at an MHC linked gene,

named *hmt* (histocompatibility dependent on maternally transmitted factor), which maps to the *Tla* region. As Mta⁺ antigen expression requires β_2 -microglobulin and Mta specific killing can be blocked by monoclonal antibodies to β_2 -microglobulin *hmt* is almost certainly a class I gene. This observation thus also explains why *mta* killing is not restricted to other MHC class I genes. □

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Invertebrate endocrinology

Steroids . . . the earliest hormones

from David L. Whitehead

ECDYSTEROIDS, the hormones controlling development in arthropods and present in certain plant families, have now been found in helminth parasites of humans and livestock (trematodes¹, filarial nematodes² and cestodes^{3,4}). As reported at a recent conference*, one result — of great potential importance to clinicians and veterinarians — is that diagnosis of *Schistosoma mansoni* infection in rodents can now be carried out by detecting ecdysteroids in the serum (using high performance liquid chromatography and radio-immunoassay). An even more exciting revelation was that injecting the infected rats with antibodies to an ecdysteroid hapten reduces the burden of worms (Philippe Nirde, Institut Pasteur, Lille). A further prospect is that use of anti-ecdysones or ecdysteroids, themselves quite harmless to vertebrates, could reduce infection by interfering with reproduction or development of endoparasites (Jan Koolman, Philipps Universität, Marburg, FRG).

A claim made in 1969 that *S. mansoni* can locate and infect snail vectors because they exude ecdysone⁵, should now be re-examined, in the light of new conclusive evidence that the pulmonates which transmit bilharzia to man contain 20-hydroxyecdysone⁶.

The function of ecdysteroids in helminths is not yet clear but it was suggested at a conference† this summer that

these polar sterols, found in most protozoa (arthropods, molluscs, annelid worms) and even some archaeocoelomata (starfish, sea anemone) arose as a signal substance rather early in evolution¹. The earliest or first chemical messenger might have been a hydroxylated cholesterol like 3 β , 14 α , 20, 22-tetrahydroxycholesterol from which ecdysteroids were then derived by a change in the β ring and further hydroxylation¹. Even gamones (for example, antheridiol, oögoniol), the steroids controlling gametogenesis in fungi, might owe their origin to this primitive hormone.

Progress in studying the biosynthesis and catabolism of ecdysteroids in arthropods was also reported at the two meetings. 22-Phosphate esters of four ecdysteroids, not bound to proteins, have been identified in the eggs of the desert locust⁷. They are known to be active conjugates because injection of labelled esters into *S. gregaria* later gives rise to free ¹⁴C-hormone in the embryos⁸ (a new phosphatase is responsible, located in the mitochondrial-lysosomal fraction). Inactive excretory metabolites (the unhydrolysable polar ecdysteroids⁹), found in well-developed embryos have been identified as ecdysoneic acid and its 20-hydroxyanalogue¹⁰, which must be formed from 26-hydroxy metabolites. 3-Acetoxyecdysone-2-phosphate is also an inactive metabolic product found in faeces of 5th instar *S. gregaria*. Just to emphasize that one should never generalize about insects, in *Pieris brassicae* larvae phosphate esters but not acetates have been found, while in the stick insect *Carausius morosus*

the situation is reversed (René Lafont, Ecole Normale Supérieure, Paris). Apparently in Diptera the 3-dehydro and inactive 3-epiecdysteroids are the predominant metabolites⁹. In *P. brassicae* and *L. migratoria* 3 α - and 3 β -hydroxy compounds were formed rapidly from 3-dehydroecdysteroids (René Lafont) suggesting that, as in vertebrates, 3-dehydro-compounds could represent active forms of the hormone in the target cell.

Considerable discussion took place concerning the evidence for a most unusual conjugate (22-AMP-2-deoxyecdysone) reported earlier to be bound to the vitellin in *Locusta migratoria* eggs¹¹. Final proof of its existence is awaited with great interest because it might, according to some laboratories, have been formed as an artefact during extraction.

A new chemiluminescence immunoassay for ecdysteroids sensitive down to 5 pg (6.2 fmol) has been developed (Lutz Reum, Marburg, FRG). The brain hormones (PTTH) responsible for directing ecdysteroid synthesis (from 7-dehydrocholesterol?) and release from the prothoracic glands of the silkworm and of tobacco hornworm have now been characterized (Hironori Ishizaki, Nagoya University, Japan and Walter Bollenbacher, University of North Carolina). Both occur in two forms. The larger (29kd) in *Manduca sexta* activates prothoracic glands *in vivo* and *in vitro* in a dose-dependent fashion. The *Bombyx* PTTH active in *Samia cynthia ricini* (1 x 10⁻¹¹M) is a single chain peptide of 40–43 residues whereas the peptide active in *Bombyx* itself is larger (~ 22kd).

Ecdysteroids in the absence of juvenile hormone act to switch off insect larval-specific genes permanently. This was very neatly demonstrated (Lynn Riddiford, University of Washington, Seattle) with the tobacco hormone caterpillar which produces a bright blue protein called insecticyanin as long as juvenile hormone continues to circulate in the blood. □

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*The Second International CNRS Symposium was held in Strasbourg at the beginning of September. The proceedings will be published in 1984.

†The VI European Ecdysone Workshop was held in Szeged, Hungary, August 23–26, 1983.

Hydrogen isotopic evidence of rhyolitic magma degassing during shallow intrusion and eruption

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Deuterium and water contents of rhyolite obsidian tephra clasts and lava flows sampled in the western USA decrease within each eruptive sequence, from D- and H₂O-rich, explosive (Plinian) eruptions, to D- and H₂O-depleted lava flows. These changes result not from volatile gradients in the magma, but rather from progressive in situ degassing; involvement of meteoric water is excluded.

KNOWLEDGE of the volatile content of magma is critical to understanding the chemical evolution of magma, the conditions in which it exists in the crust, and the mechanisms by which it erupts to the surface. The source of these volatiles (especially water) is also of interest; potential sources range from the atmosphere to the deep crust or mantle. Data concerning volatiles in magma are fragmentary because processes which result in a loss of volatiles, such as eruption and crystallization, intervene between the magmatic state and analysis of samples. Sampling of gases at active volcanic vents presents formidable logistical and analytical difficulties, particularly for the more silicic magmas^{1,2}. Thus, much of the effort to understand volatiles in silicic magmas has involved indirect approaches, such as inference from phase assemblage^{3,4}, studies of glass inclusions in crystals^{5,6}, and consideration of eruption dynamics^{7,8}.

The only volcanic materials suitable for direct analysis of retained magmatic volatiles are young, bubble-free glasses or obsidians. However, glass hydrates in a surface environment by slow inward diffusion of meteoric water which may contaminate the magmatic water. Hydration requires either a long time, as in the case of obsidian which becomes perlite, or short path length, in the case of pumice. In either case, volatiles in the sample are contaminated by a meteoric component. Friedman and Smith⁹ analysed obsidian-perlite pairs of material from rhyolite flows and domes at various locations. They found that the obsidians generally contained 0.1–0.3 wt% H₂O, which was not related in isotopic composition to local meteoric water, while the isotopic composition of water in perlite (2–4 wt% H₂O) did correlate with the isotopic composition of local waters. They interpreted these data as indicating that flow obsidian retained primary magmatic water. More recently, Eichelberger and Westrich¹⁰ found that obsidian fragments in products of explosive eruptions contain far higher volatile contents than observed in flows, and that water was the dominant volatile component (>97 wt% of total volatile content) in the clasts. Based on the high temperature of volatile release, lack of bubbles, and inability of water to diffuse into or out of the clasts on an eruptive time scale, they argued that the measured water contents represented pre-eruption concentrations of magmatic water, and that these obsidians are quenched samples of magma.

We now present the first hydrogen and oxygen isotope data for water-rich obsidian ejecta. The results provide new evidence concerning the migration of magmatic water during shallow intrusion and eruption of rhyolitic magma.

Sample selection

To avoid the problem of hydration and to obtain high-quality glasses, we have restricted this initial study to very young (≤2,000 yr) rhyolite volcanoes. Friedman¹¹ has shown that glasses of this age in climatic conditions of western United States

develop hydration rinds no more than a few micrometres thick on old, exposed surfaces. As far as possible, we have removed outer surfaces from the samples before analysis. However, analyses of core and old surface pairs from individual fragments showed no detectable difference in water content.

Eruption sequences sampled are tephra and lava from the Big Obsidian Flow vent¹², Newberry Caldera, Oregon; from Glass Mountain and Little Glass Mountain vents¹³, Medicine Lake Highland, California; and from vents at Glass Creek of the Inyo Domes chain^{14,15}, Long Valley Caldera, California. All of these vents produced extensive Plinian air-fall deposits, which were followed by extrusion of obsidian flows. Volumes of magma erupted in each tephra plus lava sequence are of the order of 1 km³ in each case.

Obsidian occurs in air-fall tephra at these sites as clasts ranging in size up to ~10 cm, and as rinds on bread-crust pumice bombs. The obsidian clasts were sampled from sections through sequentially-erupted multiple deposits of tephra located close to the source vents, and exposed by hand digging. Stratigraphical position within these sections, plus observed basal contact relationships of tephra sections, provided the relative age for each sample. (Deposits from areas studied are further described in refs 10, 12–15.) Samples analysed were bubble-free or nearly bubble-free individual clasts ≥1 cm. In flows, obsidian is found as a central zone between an upper pumice zone 5–10 m thick and a lower pumice zone of variable thickness¹⁶. Again, bubble-free material was sampled.

Analytical techniques

Water content was determined by a Dupont moisture analyser, using a P₂O₅ cell across which an electrical potential is applied. Water released from the sample in a furnace is carried into the cell by He gas and absorbed, causing the cell to become conductive and the water to be electrolysed. The quantity of current, integrated over time of the run, required to electrolyse the absorbed water is recorded. Standards, consisting of water-filled capillaries of known volume, and blanks are run before and after each group of 4–6 samples. Preparation procedures were described previously¹⁰. For samples ground to <75 μm, heating to 800 °C for 20 min is sufficient to release all the water. Replicate analyses commonly yielded a standard deviation ≤3% relative to the concentration. Analyses of USGS standards and hydrated salts yielded water contents which deviated ≤3% from accepted values.

Water was extracted from obsidian for hydrogen isotope analysis by melting *in vacuo*, after degassing the samples under high vacuum at 150 °C for a minimum of 2 h, and generally overnight. The induction heater and vacuum apparatus used were similar to those described by Friedman and Smith⁹. Before installation of a Toepler pump, we used activated charcoal

Table 1 Oxygen and hydrogen isotope compositions and water contents of obsidian clasts and flows

Sample	H ₂ O (wt%)	$\delta^{18}\text{O}$	δD	Sample	H ₂ O (wt%)	$\delta^{18}\text{O}$	δD
Mammoth Lakes				GML-1 (flow)	0.26	+7.62	-131.2
Glass Creek				GML-2 (flow)	0.12	+7.42	-118.8
82-I-3A-3	2.04	+7.79	-61.8	GML (flow)	0.12		-121.7
-6	1.74	+8.03	-63.5				
-1	1.55	+7.74	-51.1	Medicine Lake			
-9	1.44	+8.03	-58.3	Little Glass Mountain			
-2	1.41	+8.02	-52.2	LGM-5A-6	3.12	+7.46	-68.6
-8	1.09	+8.14	-63.1	LGM-5A-1	2.21	+7.55	-64.8
-4	1.07	+7.87	-67.4	LGM-5A-8	2.20		-67.3
-10	0.75	+7.70		LGM-5A-2	2.05	+8.66	
-5	0.66	+7.94	-75.6	LGM-5A-5	2.01	+7.87	-67.4
82-I-1C Rind	1.12		-87.4	LGM-5A-7	1.79		-73.0
82-I-50-BT-1 (pumice)			-95.3	LGM-5A-3	1.46	+7.81	-73.5
-A (flow)		+8.00	-106.1	LGM-a-1	1.17	+7.34	-66.7
-C (flow)		+10.67	-114.7	LGM-5C-3	1.04	+7.72	-82.6
-E (flow)	0.27	+8.04	-118.5	LGM-5C-6	1.25		-77.6
-F (flow)	0.27	+8.82	-120.5	LGM-5D-6	0.93	+7.46	-82.3
-G (flow)	0.27	+7.90	-115.9	LGM-5D-1	0.79	+7.73	-73.6
-H (flow)	0.23	+7.78	-123.5	LGM-5D-2	0.73	+7.92	-80.7
-I (flow)		+8.09	-113.6	LGM-5D-3	0.62	+7.71	-86.3
-J (flow)	0.23	+7.85	-129.8	LGM-5D-4	0.61	+7.75	-84.6
-K (flow)				LGM-5E-5	0.92	+8.28	-82.9
Medicine Lake				LGM-5E-2	0.81		-74.5
Glass Mountain				LGM-1I	0.44	+8.02	-88.5
BGM-5-1	1.93	+7.61	-63.3	LGM-2F (flow)	0.15	+7.52	-127.4
BGM-5-4	1.27	+7.41	-68.3	LGM-6 (flow)	0.07		-128.7
BGM2-I-4	0.93	+7.38	-80.4				
GM-2B2-0	0.89	+7.50	-65.7	Newberry Crater			
GM-2C2-0	0.78		-63.7	NC-5-V	0.91	+7.28	-63.4
BGM-2-I-5	0.60	+7.52	-90.0	NC5-VII-I	0.47	+7.04	-83.6
BGM9-II-7	0.41	+8.46	-86.9	NC-3	0.12	+6.96	-128.9

(degassed at 200 °C under high vacuum at least 12 h) at liquid nitrogen temperature to collect the H₂ from several samples. Molybdenum crucibles were used and the non-condensable volatiles were exposed to CuO heated to 450 °C to assure quantitative collection of all hydrogen as H₂O. Because small samples (≤ 30 μmoles) of hydrogen can suffer isotopic fractionation using the charcoal adsorption method, sufficient sample was melted to yield at least 50 μmoles of H₂.

Isotopic analysis of the hydrogen was performed in a modified Varian GD 150 double collecting ratio mass spectrometer. Correction for H₃⁺ was determined by analysing both V-SMOW and GISP International Standards against a laboratory working standard. Reproducibility of δD values was $\sim 1.0\%$.

Oxygen isotope compositions of obsidian were determined on CO₂ prepared from oxygen released by fluorination of the samples with ClF₃ (ref. 17) at 575 °C in nickel reaction tubes fitted with caps designed for rapid, contamination-free sample loading. The oxygen was converted to CO₂ by reaction with a 2-cm segment of spectrographic-pure graphite rod held in a resistance heated platinum wire coil. $\delta^{18}\text{O}$ of meteoric water was determined by equilibration with CO₂ using a fractionation factor of 1.0412. Isotopic analysis of the CO₂ was performed in a modified Nuclide 6", 60° double collecting ratio mass spectrometer. Our average $\delta^{18}\text{O}$ value for NBS no. 28 is +9.64‰, with a reproducibility usually better than 0.1‰.

Results

Oxygen isotope compositions of obsidian show little variation within eruption sequence (Table 1). Most $\delta^{18}\text{O}$ values lie between +7.0‰ and +8.0‰, which are generally regarded as primary magmatic values¹⁸. In contrast, both the water content and hydrogen isotope composition of obsidian show large variations within individual eruptive sequences, and are related to mode of eruption (explosive versus non-explosive) and the

relative age of each obsidian sample in the eruption sequence. Water contents below 0.4 wt% are seldom observed in obsidian from tephra, and values in Table 1 are as high as 3.1 wt%, more than a factor of 3 greater than previously reported for fresh volcanic glass (exclusive of glass inclusions in crystals). Water contents > 0.4 wt% are rare in obsidian from flows, and values close to 0.2 wt% are more common. Water in the tephra obsidian is much richer in deuterium than water in obsidian from the flows. δD varies from -51 to -90‰ in tephra, and from -106 to -131‰ in flows. No correlation is seen with the isotopic composition of local meteoric water (Table 2).

A marked decrease in δD is associated with a decrease in water content in the suite of samples from Little Glass Mountain (Fig. 1). Qualitatively similar relationships have been reported for granitic rocks^{19,20}, although these rocks must record a more advanced stage of magmatic evolution considering their more extensive crystallization histories. Figure 2 shows a general decrease in the water content of obsidian from Little Glass Mountain, from beginning to end of eruption. Additional samples in Table 1 amplify the trend shown in Fig. 2; initial water contents as high as 3.1% H₂O are also indicated. Thus, within a single eruption sequence, there is a decline in water content, δD , and explosiveness of eruption with time.

Similar variations in δD and water content found for the LGM series of samples (Fig. 1) are exhibited by glasses from other eruptive sequences and volcanic centres, as shown in Fig. 3. Thus, a decrease in water and in deuterium content of rhyolitic magmas with time during an eruptive sequence appears to be a general phenomenon among the examples studied.

Interpretation

Because of the low diffusivity of water in glass¹¹, measured water contents and isotopic compositions of fresh obsidian represent the water contents and isotopic compositions at the time the material cooled. Because obsidian in tephra cooled before,

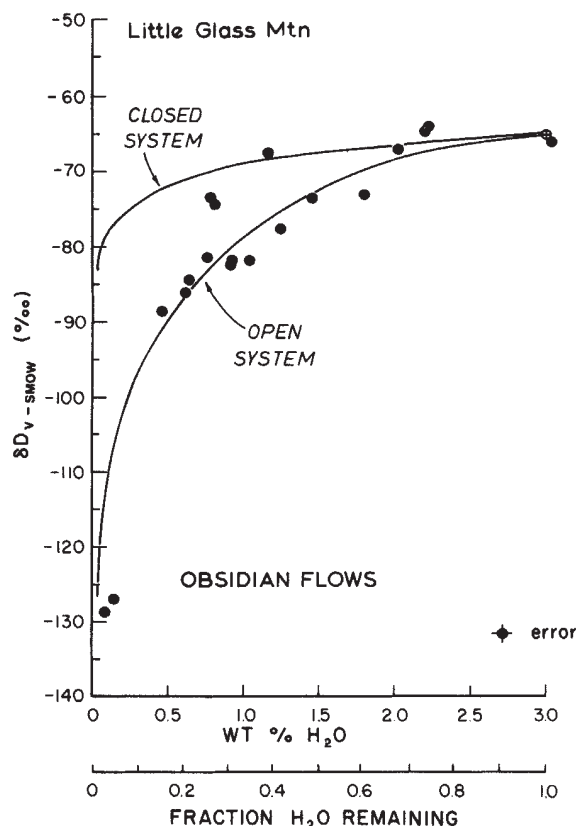


Fig. 1 Obsidian clasts from air fall tephra layers and obsidian flows at Little Glass Mountain exhibit a marked decrease in hydrogen isotope composition with wt% H_2O . Open- and closed-system Rayleigh distillation curves are plotted for comparison, assuming an initial δD and wt% H_2O of -65% and 3.0% , respectively, and a water-melt hydrogen isotope fractionation factor of 1.004. Fraction H_2O remaining, refers to the water content of the silicate melt. Error bar represents approximate analytical uncertainty at the 2σ -level.

or within tens of seconds during eruption¹⁰, the analyses represent pre-eruption compositions. In the case of obsidian in flows, cooling occurred primarily after emplacement and the analyses represent postemplacement compositions. While it cannot be established with certainty that obsidian within an eruptive unit is chemically representative of the erupted magma as a whole, it is identical to pumice in anhydrous composition and forms a volumetrically significant portion of the deposits¹⁰. Therefore, the results indicate a decline with time in water content of magma involved in the eruptions.

Several workers^{21,22} have suggested that an upward increase in volatile content should be characteristic for silicic volcanic magmas. Chemical zonation associated with these magmas has been ascribed to diffusional transport via volatile complexing which might also produce a roofward-increase in volatiles. No detectable variation in major or trace element chemistry has been found to date in several sequences of obsidians (Stockman and Westrich, personal communication). In addition, the data in Table 1 give no evidence of any oxygen isotope zonation within those parts of the magma chambers represented by our samples. It is tempting to ascribe the progressive decrease in water content in our samples to the tapping of drier magma at deeper levels.

Degassing of magma during the eruptive sequence implies the presence of a vapour phase. Generation of a free vapour phase within a magma provides the possibility that the hydrogen isotope composition of the newly formed vapour will be different from that of water still dissolved in the magma. No experimental data are available concerning isotopic equilibrium between hydrous silicate melt and vapour. However, for most hydrous

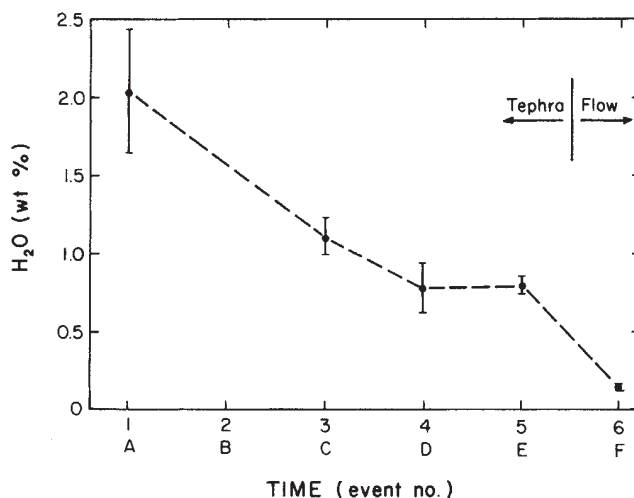


Fig. 2 Analysis of a carefully selected suite of samples from Little Glass Mountain (LGM), Medicine Lake Highlands demonstrates the decrease in water content of glass with time during an eruptive sequence. The time axis is schematic. LGM samples in Table 1 are designated with the letters of 'events' shown here. Bars represent 1σ -variation in water content of different samples; dots show the mean values ($n = 10$). No obsidian clasts occurred in layer B. Not all of the samples represented here were analysed for their hydrogen isotopes.

Table 2 Range of isotopic composition of local meteoric waters from or near Medicine Lake Highlands, Newberry and Mammoth Lakes volcanic areas

Location	$\delta^{18}O^*$	δD	Ref.
Mammoth Lakes	-15.8 to -16.1	-121 to -124	This study
Medicine Lake Highlands	-12.1 to -12.9	-92 to -98	34
Newberry Caldera	-15.8 to -16.1	-121 to -124	35

* Calculated from $\delta D = 8\delta^{18}O + 5$.

silicate minerals at magmatic temperatures, deuterium is partitioned into the vapour²³ and the fractionation effect for oxygen isotopes is small^{18,19}. If, as might be expected, the situation is similar for hydrous silicate melt and vapour, then loss of vapour at magmatic temperatures will progressively deplete the magma in water and deuterium²⁰, without significantly affecting the oxygen isotope composition.

The effect of degassing on the hydrogen isotope composition of the magma can be calculated given the initial water content and its hydrogen isotope composition, a fractionation factor and an assumption regarding the degree of continued equilibrium between melt and vapour. We assume an initial water content of 3 wt% and δD of -60 to -70% , corresponding to the wettest obsidians we have found. A hydrogen isotope fractionation factor between vapour and melt of 1.004 is used in our calculations, and lies within the range of factors based on experimental data for crystal-vapour systems of Suzuoki and Epstein²³, but differs from the high-pressure melt-vapour data of Kuroda *et al.*²⁴. The effect of pressure on hydrogen isotope fractionation is not known and is neglected. The endmember cases: (1) no isotopic exchange between melt and exsolved vapour after vapour separation (open system) and (2) maintained equilibrium (closed system) are plotted in Fig. 1. Most of the data are well described by open system fractionation. The fact that the compositions of obsidian flows in Figs 1 and 3 plot slightly to the water-rich side of the open-system curve suggests a lower temperature (and therefore larger fractionation factor) for degassing of flow than for tephra-obsidian. For the assumed initial compositions, the hydrogen isotope compositions of the obsidian flows cannot be generated through closed system behaviour. We

therefore conclude that open-system degassing is the dominant process which has produced the observed compositional variations.

Diffusivities of H_2 in rhyolitic melts are poorly known, but are probably greater than for H_2O . The large isotope fractionation between H_2 and H_2O at magmatic temperatures²⁵ would produce isotopic variations opposite to those observed (Figs 1 and 2). Diffusion of H_2O out of the melt would deplete the magma in water, but would not produce the large isotopic changes observed; a discrete H_2O -vapour phase is required. Contamination with meteoric water can also be ruled out based on the primary $\delta^{18}O$ values of the obsidian (Table 1), and the fact that absorption of meteoric water (see Table 2) would produce a decrease in δD , but an increase in wt% H_2O . Thus, the isotopic and chemical data are best interpreted as the loss of hydrogen from the melt in the form of an H_2O vapour phase.

The hydrogen isotope composition of the water evolved will, of course, be governed by the isotopic composition of the melt, which changes as water exsolves. Depending on the nature of the 'plumbing system' controlling migration of water vapour from the volcanic conduit, the δD value of the vapour will be on or between curves for open and closed system examples shown in Fig. 3. Vapour which collects in a reservoir will change in δD only slightly, as shown for the closed system case. Vapour which continually leaves the magma and volcanic conduit, however, may vary greatly, decreasing in δD with time as the magma evolves, as shown in Fig. 3 for the open system case. Water vapour sampled above a volcanic conduit could thus have a δD value more characteristic of some meteoric waters than magmatic water (see Table 2).

Degassing

The available data provide some unique constraints regarding how degassing occurs. Most of the degassing takes place on the time scale of the eruptive sequence. This is evident from the isotopic data which indicate that the magma represented by each successive tephra deposit was degassed to a greater degree than previously erupted magma. Vapour saturation, required for loss of H_2O , and detected by a shift in the hydrogen isotope composition of the quenched magma (obsidian), is evident from the earliest tephra deposit. The similarity of δD values of the obsidians to the 'magmatic' range¹⁸ permits only relatively minor loss of H_2O vapour before eruption of the earliest tephra deposit. From the lack of evidence for erosion or soil development between tephra layers, and from observations of modern tephra-producing eruptions^{26,27}, the time scale of degassing described here must be of the order of years or less. An additional time constraint is implied by the low water contents of the obsidian lava flows. Based on experimentally measured water solubility in rhyolite obsidian at 1 atm water pressure²⁸, the lowest water contents in Table 1 require degassing in essentially surface conditions. Obviously, magma cannot remain in a molten state in such conditions for very long. This short time scale requires a mechanism for the rapid migration of water in a shallow silicate melt. The rise of bubbles of observed sizes in rhyolitic magma is negligible on a time scale of years. Because of the low diffusivity of water in melt, even considering variation of diffusion with water content²⁹⁻³¹, diffusion can only occur over short distances on a time scale of years³². The only process which can quickly extract large volumes of vapour from rhyolitic magma is leakage of gas from regions in the magma that at times resemble a permeable magmatic foam. This involves diffusion of water through the melt comprising the 1–10- μm thick bubble walls, followed by flow of water vapour through interconnected (coalesced) bubbles and out of the system. The process is illustrated on a small scale by pumice clasts. Because such clasts obviously no longer retain as trapped gas all the vapour they contained at fragmentation, they must have leaked.

Samples analysed in this study contained very few or no bubbles. To have degassed as inferred here, the obsidian must have contained bubbles in the past sufficient in quantity to form a permeable foam. Because the bubbles could not be lost by

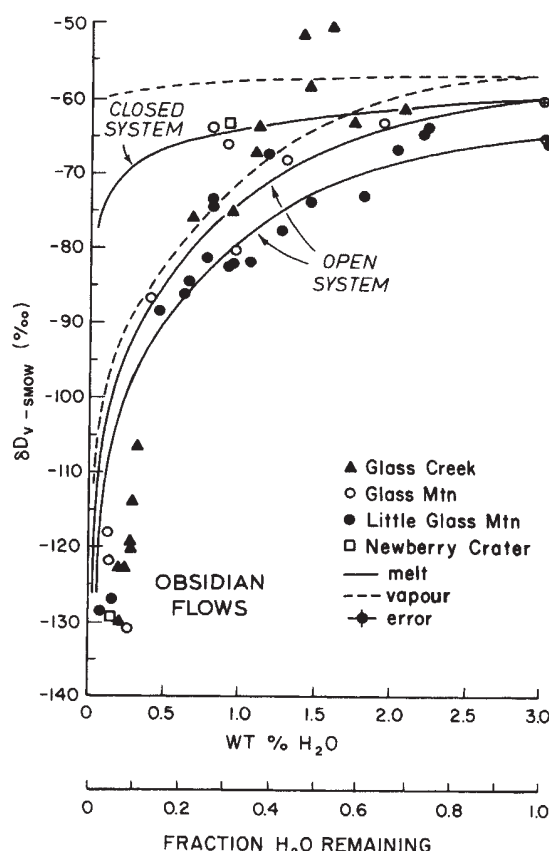


Fig. 3 Samples from a number of volcanic centres illustrate a decrease in δD with wt% H_2O similar to that shown in Fig. 1 for the LGM series. Rayleigh distillation curves are drawn for comparison, beginning with melts having an assumed δD initial of -60 and -65% . Dashed lines indicate the isotopic composition of water exsolved in the open- and closed-system cases. Assumptions are those for curves in Fig. 1.

migration, they must have been resorbed. Complete resorption of bubbles in vassicated, degassing magma is possible provided that discharge of gas is rapid compared with bubble collapse²⁸. This allows vapour pressure to drop to a value less than lithostatic load before bubble collapse closes the system to gas flow. The melt would be locally undersaturated in H_2O , causing vapour to be resorbed as pressure equilibrium is re-established. Collapse of voids in rhyolite magma may be a slow process as welded zones in ash flow tuffs are restricted to portions of cooling units thought to remain at magmatic temperatures for weeks or months³³, although flow of the magma would tend to accelerate collapse and destruction of bubbles. Further discussion of this process, which is analogous to welding in tuffs, and supportive isotopic evidence will be presented in a subsequent paper on obsidian flows.

We thus infer that the obsidian clasts represent quenched, variably degassed magma. As the degassing occurred before eruption of the material, we conclude that a significant proportion of the volatiles contained in rhyolitic magma are released in the conduit rather than on explosive fragmentation during eruption. It is this gradual leakage at depth which eventually produces magma dry enough to erupt without fragmentation as flows or domes. If the water contents observed in obsidian tephra clasts are representative of pre-eruption concentrations of the magma as a whole, then degassing to ~ 0.5 wt% water is required for extrusion of a flow or dome. Indeed, obsidian quenched before eruption (as opposed to welded after emplacement) on the outer portion of a dome associated with Inyo volcanism has been found to contain 0.5 wt% water. Once extruded on the surface as a flow or dome, the magma degasses to a vapour pressure of 1 atm. Ultimately this results in obsidian with $\delta D \leq$

115 and 0.2 wt% H_2O , the latter consistent with the experimentally measured solubility of water in melt under 1 atm water vapour pressure²⁸.

Thus far, δD is the only compositional parameter which we have found to vary significantly with water content within obsidian suites from a single eruptive sequence. Crystal content, which might be expected to increase with decreasing water content, remains low and in some sample suites crystals are virtually absent. In view of the isotopic evidence for degassing, uniformly low crystal contents throughout eruptive sequences have two important implications: (1) low water (and low δD) melts are metastable (that is supercooled due to loss of water) when erupted, and (2) second boiling phenomena do not contribute to bubble growth during eruption. Vapour exsolution and subsequent explosive eruption of these magmas are apparently solely due to decompression during upward intrusion.

The hydrogen isotope systematics described here should also apply to subvolcanic regimes. The exsolution and loss of a free vapour phase would thus explain the hydrogen isotope data of O'Neil *et al.*¹⁹ on porphyritic rocks in Australia, and corroborate the interpretation of Nabelek *et al.*²⁰ of hydrogen isotope variations in a high level granitic pluton in Nevada. The hydrogen isotope composition of magmatic water retained by the magma is dependent on the magma's degassing history.

Conclusions

Hydrogen isotope and water analyses of obsidian from several rhyolitic eruption sequences indicate that progressive, *in situ*

degassing of magma occurs at depth beneath the volcanoes during the eruptive sequence. This degassing appears to be a necessary prelude to non-explosive eruption of magma as flows or domes. Initial (pre-eruption) magmatic conditions consistent with the data are 3 wt% H_2O and $\delta D = -60$ to -65% .

Fractional distillation of the magma column can produce rhyolites with δD values as low as -130% (wt% $H_2O \approx 0.2$) without any detectable interaction with meteoric water. Water exsolved from a melt in a volcanic conduit may also show a large depletion in deuterium, with δD becoming more negative with time. Hydrogen isotope ratios of H_2O in fumarolic gases on active volcanoes may thus serve as a measure of magma evolution. Water vapour with δD values more negative than the -40 to -80% range accepted for magmatic water¹⁸ may be released from the melt and these δD values do not necessarily indicate incorporation of meteoric water.

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Formation of fossil hydrothermal chimneys and mounds from Silvermines, Ireland

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Sulphur isotope analyses reveal that the fossil hydrothermal pyrite mound and tubes at Silvermines were produced by the reaction of hydrothermal Fe(II) with bacteriogenically-reduced sulphur species ($\delta^{34}S$ range: -18.4 to -42.5%). This took place in a brine pool dominated by hypersaline Carboniferous seawater. This is in contrast to their modern, but considerably larger, morphological counterparts forming by direct precipitation of hydrothermal sulphides on the East Pacific Rise at $21^\circ N$.

THE Silvermines orebodies in Central Ireland consist of three contiguous sedimentary zones, two of which (the Mogul 'B' and Upper 'G' zones) are pyritic zinc + lead deposits, while the third

(the Magcobar deposit at Ballynoe) is composed of baryte. They occupy one horizon in a Lower Carboniferous succession of unmetamorphosed and only slightly deformed carbonates¹⁻³. That they were laid down as chemical sediments is beyond doubt¹⁻⁴, the most telling evidence being the occurrence of plastically deformed sedimentary sulphide (S. Taylor, personal

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Fig. 1 Example of a 360-Myr old hydrothermal chimney from the Silvermines Zn+Pb+Ba deposit (8 mm chimney diameter).

communication) and jasper intraclasts as components in the ubiquitous submarine debris flow deposits found within, and just above the orebodies. Their age, deduced from their setting, is late Tournaisian, 360 Myr (ref. 5).

Cross-cutting veins of galena(PbS) + sphalerite(ZnS) + baryte(BaSO_4) + quartz are clearly spatially and genetically related to the sedimentary ores: they mark the conduits that channelled the solutions from depth to the early Carboniferous sea floor^{3,4,6}.

We know from recent discoveries at 21° N on the East Pacific Rise (EPR) that as hot ($\sim 350^\circ\text{C}$)⁷, metal-bearing aqueous solutions exhale from oceanic crust into near freezing seawater, immediate precipitation of sulphides by quenching leads to the formation of chimney spires (originally anhydrite⁸) and mounds composed, now, essentially of pyrrhotite (Fe_{1-n}S), pyrite (FeS_2), chalcopyrite (CuFeS_2) and sphalerite (ZnS) or wurtzite (a sphalerite polymorph)^{9,10}. The $\delta^{34}\text{S}$ of the pyrite is relatively uniform, ranging from +1.4‰ to +3.0‰ (ref 11). (Figure 4a shows the full range of S-isotope results from EPR chimney sulphide.) It has been demonstrated that $\sim 90\%$ of the sulphur was released from basaltic crust during hydrothermal metamorphism by the circulating seawater–hydrothermal fluids¹¹.

The submarine hot springs that fed the Silvermines exhalative sulphide and baryte orebodies should have produced similar structures, an expectation satisfied by Larter *et al.*⁴ who discovered a pyrite mound and small chimneys in the Ballynoe baryte deposit (Fig. 1). The Silvermines chimneys, although morphologically comparable to those at 21° N on the EPR, have their own distinct character. The individual chimney spires have a smaller diameter (EPR ranging from 0.5 to 400 mm^{7,10} whereas Silvermines are generally ≤ 20 mm⁴) and are composed almost entirely of pyrite. These differences presumably relate to the marked contrasts in their marine and tectonic settings. The wider chimneys of the EPR are constructed at a seawater depth of 2.5 km as products of the exhalation of a vigorous hydrothermal convective system. The circulating fluid is dominantly seawater¹¹, modified by reaction with, and driven by heat from solidifying magma intruded at a constructive plate margin¹². Iron, copper and zinc sulphides are appreciably soluble in these conditions¹³. At Silvermines the hydrothermal system was probably driven by heat within the continental crust¹⁴ and was consequently of lower vigour and the discharge rate proportionally lower. Moreover fluid inclusions indicate that the temperature of the effluent was certainly $<150^\circ\text{C}$, having been cooled from $\sim 250^\circ\text{C}$ by shallow crustal mixing with a solution of higher salinity (20% NaCl equiv. wt% compared with primary solution around 12% NaCl equiv. wt%). The most likely candidate for the dense brine is highly saline seawater¹⁵, produced by evaporation on the margin of the fault-controlled basin¹⁶ which occupied the area of Silvermines during the Lower Carboniferous.

We report here the results of a sulphur isotope study on this first discovery of fossil hydrothermal mound and chimneys. Although we expected further contrasts with the EPR chimneys to be revealed, we were not prepared for the remarkably light values of $\delta^{34}\text{S}$ ‰ in some of the tubes, a factor that requires extreme bacteriogenic fractionation of sulphate (Table 1). These unusual values may help us to identify the feeder zones in other sediment-hosted exhalative zinc+lead deposits.

New finds and descriptions

In the EPR hot spring areas, the vents are invariably found in mounds, which comprise accumulations of mineral debris and chimney clusters. Each EPR mound covers an area of 15×30 m with a height of 2–10 m^{9,17}. The remnants of a comparable mound have now been recognized in the south-east corner of the Ballynoe open-pit baryte deposit, where the surface is essentially a silicified footwall pavement (Fig. 2). Although this area has been damaged by heavy plant excavators, distinctive features can still be recognized. The most convincing relic is a massive lenticular body, 3×8.5 m in plan and up to 2 m high (10 m E of 10500 N, 10900 E, Fig. 2). It consists exclusively of pyrite at its base with an increasing baryte content towards the top. Although most of the pyrite is massive or colloform there is one outcrop near a discontinuity on the east edge of the open pit where large (≤ 40 mm) pyrite cubes and tabular baryte crystals (≤ 30 mm across) have been developed, presumably as a result of continued chemical reworking of the mound by hydrothermal fluids (black ornament, Fig. 2). Around this mound is a mappable development of pyrite, with minor baryte, exhibiting textures akin to those in the mound and chimneys, varying from 0.01 to 1.5 m thick (fine stipple, Fig. 2).

Well developed chimneys were described by Larter *et al.*⁴ as being tubular (0.1–20 mm in diameter), and comprising concentric pyrite layers (0.05–1 mm thick), suggestive of Liesegang rings; and an outer coating of crystalline, tabular baryte laths, termed peripheral baryte (≤ 10 mm in length). The matrix between these vents is dominantly barytic. These we term type I chimneys and rods.

Type II chimneys are similar to type I in that they exhibit a tubular form, are composed mainly of concentrically zoned pyrite, and are about the same size: they differ in several aspects. Instead of the central canals comprising framboidal pyrite⁴, the type IIa vents either have a central infilling rod composed of white/grey translucent sphalerite (cleiophane), coated with microcrystalline pyrite, or are empty. Framboidal pyrite is generally less abundant in the outer zones where radiating acicular pyrite crystal dominates. Baryte is a minor associate of type II vents: peripheral baryte has not been found. Instead, where chimneys do not abut, they terminate with an outer layer of euhedral to subhedral pyrite and sphalerite. These textures are reminiscent of a crystal garden. The structures appear to have begun life as thin colloform developments or tubular films, grown from colloidal FeS_2 , with diffusive and osmotic processes increasing their thickness and length. As with type I vents, type IIa tubes come from the Ballynoe baryte deposit; type IIb tubes come from the Mogul zinc+lead deposit, but are essentially similar to type IIa (see also ref. 18). Only type IIa tubes have been found *in situ*.

Isotopic results

All isotopic analyses were obtained using standard techniques^{19–21}, and are reported as $\delta^{34}\text{S}$ ‰ differences relative to the Cañon Diablo Troilite (CDT) standard. Analytical uncertainty is about 0.3‰—the difference between duplicate complete analyses.

Baryte results are bimodal around +14.2‰ and +20‰ (Table 1). All pyrite ratios are isotopically very light, particularly the outermost zones of the type I vents. The lightest value from these zones, -42.5 ‰ (sample Bal-10), is the most fractionated sulphide result reported from any Irish ore deposit. Type I pyrite results show a notable variation of ~ 10 ‰ from outer through

Table 1 S-isotope results of the hydrothermal chimneys and rods from the Silvermines Zn + Pb + Ba deposit

Chimney type	Laboratory no.	Sample no.	Mineral	Sample site	$1\delta^{34}\text{S}_{\text{CDT}} (\text{‰})$
I	S 3493	AB -5	Pyrite	Central zone	-31.3
I	S 3960	BAL -1	Pyrite	Central zone	-33.5
I	S 3492	AB -4	Pyrite	Central and inner zones	-38.8
I	S 3494	AB -1	Pyrite	Central and inner zones	-38.2
I	S 3958	BAL -9	Pyrite	Central and inner zones	-38.1
I	S 3959	BAL-10	Pyrite	Outer zones	-42.5
I	S 3977	BAL -7	Pyrite	Outer zones	-40.4
I	S 3495	AB -2	Baryte	Peripheral	+19.2
I	S 3496	AB -3	Baryte	Peripheral	+20.4
I	S 3962	BAL -3	Baryte	Peripheral	+14.2
I	S 3964	BAL-11	Baryte	Peripheral	+14.2
I	S 3982	AB -6	Sphalerite	Matrix to chimneys	-18.9
I	S 3938	BAL -4	Sphalerite	Matrix to chimneys	-20.6
IIa	S 3954	BPU-10	Pyrite	Innermost zone	-21.2
IIa	S 3955	BPU -5	Pyrite	Innermost zone	-19.0
IIa	S 3957	BAL -8	Pyrite	Innermost zone	-21.4
IIa	S 3960	BPU -2	Pyrite	Innermost zone	-20.2
IIa	S 3999	BPR -1	Pyrite	Innermost zone	-18.4
IIa	S 4000	BPR -4	Pyrite	Mid zones	-20.2
IIa	S 4001	BPR -6	Pyrite	Mid zones	-19.4
IIa	S 3961	BPR -3	Pyrite	Outer zones	-18.7
IIa	S 4002	BPR -3	Pyrite	Outer zones	-19.9
IIa	S 3978	BAL1-1	Pyrite	Outer zones	-23.7
IIa	S 4318	BPI-01	Pyrite	Botryoidal cap to chimney	-21.1
IIb	S 3990	Mogul Pipe	Pyrite	Inner zones	-17.2
IIb	S 3991	Mogul Pipe	Pyrite	Outer zones	-11.6

to inner zones (Fig. 3). The $\delta^{34}\text{S}$ in sphalerite associated with these vents is considerably heavier.

All analyses on type IIa vents were of pyrite since this was the only mineral available in sufficient quantity for this technique. Unlike type I, these results show no significant variation across the zones: they give an average value of -20‰. Type IIb vents give results of -17.2‰ (inner) and -11.6‰ (outer). The significance of these type IIb results will not be discussed until more substantial data can be collated.

Previous S-isotope studies

Two S-isotope studies have been carried out on these deposits. Concentrating on the Lower 'G' discordant and Upper 'G' stratiform orebodies, Greig *et al.*²² interpreted the inter-mineral variations of $\delta^{34}\text{S}$ in terms of changes in isotopic equilibria between S-isotopic species in the exhaling fluids. These changes were held to be the result of variations in f_{O_2} (fugacity), pH and temperature, fashioned by host rock-hydrothermal solution reactions, as the mineralizing fluids passed through the rock pile via the Silvermines fault system.

The validity of much of this interpretation is now in doubt. The hypothesis was grounded in the epigenetic, replacement model for the genesis of the Upper 'G' and the baryte zones. Myriad sedimentary features in and associated with the ore have shown this model to be untenable^{1-4,23-25}. Thus an alternative interpretation of the data is required. For example, instead of the $\delta^{34}\text{S}_{\text{baryte}}$ from the Ballynoe deposit being the product of equilibrium isotopic exchange in a fluid (that is, oxidation of reduced sulphur), it is now considered to have formed by reaction of exhaled Ba(II) with contemporaneous Lower Carboniferous seawater sulphate^{1,4,6}. Hence, the S-isotope ratio of the baryte is indistinguishable from the range of values proposed for Lower Carboniferous seawater sulphate, that is $\delta^{34}\text{S} = +14$ to $+22\text{‰}$ (ref. 26). (Note the Lower Carboniferous seawater sulphate analyses show a greater inter-basinal spread of $\delta^{34}\text{S}$ than most other geological periods²⁷.) As more data have become available on the conditions of mineral deposition at Silvermines, it is clear that disequilibria between all phases were pervasive in the upper, sedimentary ores; and that sulphate was out of equilibrium with sulphide in the lower, epigenetic ores.

Nonetheless, we agree with the conclusions of Greig *et al.*²², that fractionations between sulphide species in the Lower 'G' replacement orebody approach equilibrium, and concur that the sulphur was derived from depth, with a $\Sigma^{34}\text{S}$ of around +3‰.

In the second study, Coomer and Robinson⁶ reinterpreted the S-isotope data based on a hydrothermal exhalative model, in which ore precipitation occurred both in epigenetic-type veins, and in sedimentary lenses on the sea floor. This basis is satisfactory in the light of the discovery of the hydrothermal chimneys described above. They suggest that a mixed source for the sulphur in the sulphide was available. The first is a deep-seated source similar to that suggested by Greig *et al.*²², which precipitated minerals, particularly galena and sphalerite, at or near equilibrium in changing physicochemical conditions. Such a source of sulphur accords with the isotopic characteristics of the vein as well as the replacement epigenetic deposits, like the Lower 'G' zone. The second sulphide-sulphur source was derived from the low temperature bacteriogenic reduction of seawater sulphate, an explanation consistent with the bulk of S-isotope ratios of the Upper 'G' and 'B' orebodies.

Combining results from both studies and comparing them with the chimney isotope data (Fig. 4b) shows that type IIa tubes are broadly comparable with the average pyrite values for the Silvermines sedimentary deposits, whereas the type I vents have notably lighter ratios. Baryte results span approximately the same range, but sulphate in the orebodies does not exhibit a discernible bimodal distribution.

Genetic model

The isotopic results of type I chimneys are particularly unusual: the lightest value, -42.5‰, being extraordinary in most comparable geological environments.

We discuss a genetic model for both types of tube based on the sulphur isotope results, the fluid inclusion data¹⁵, and the geological environment as determined from the tectonics and sedimentology of the area¹⁶. Fluid inclusion studies indicate temperatures of exhalation below 150 °C. This is, of course, in marked contrast to the issuing temperatures of 350 °C indicated for the EPR black smokers. Fluid inclusion salinity analyses¹⁵ on peripheral baryte, and a sample of baryte from the Ballynoe

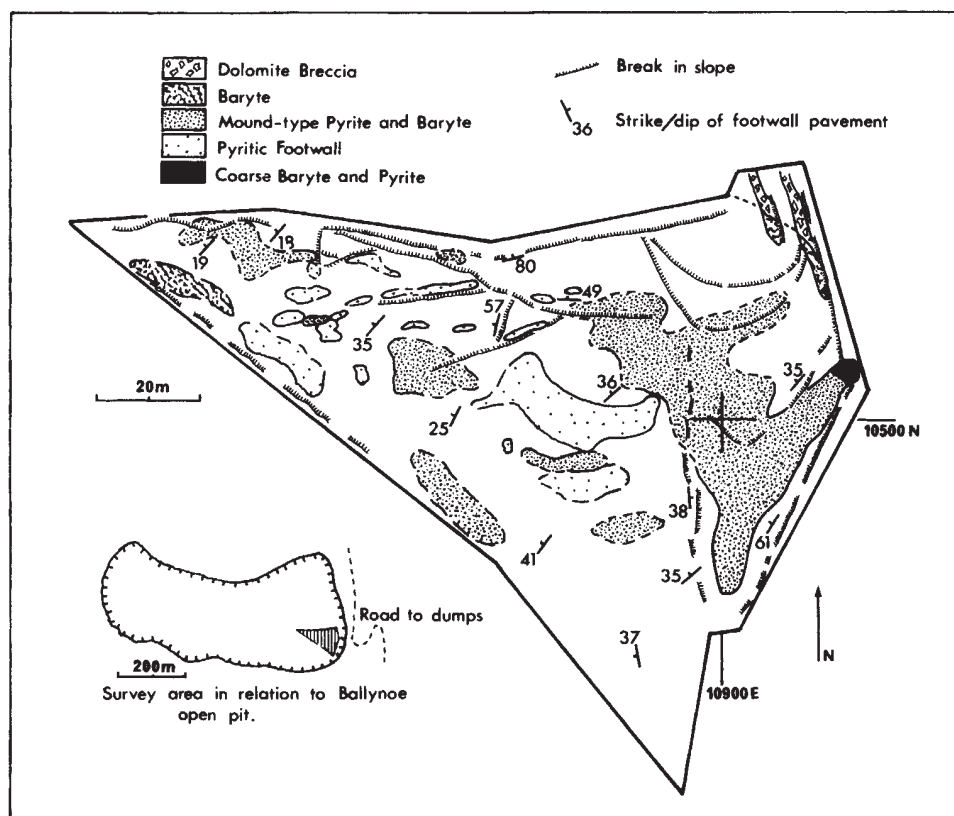


Fig. 2 Detailed map of the remains of a hydrothermal mound site in the Ballynoe open pit baryte deposit. The area is on generally northward dipping silicified footwall (no ornament).

deposit, which showed early diagenetic recrystallization of baryte causing cusping of still-soft pyritic mud (fig. 4 of ref. 16), indicated that the fluid at, and just beneath the sediment-seawater interface had an 18–22% NaCl equiv. wt% salinity. This compares with an ~12% NaCl equiv. wt% salinity in the primary mineralizing solutions. We suggest the former fluids reflect the presence of a brine pool in the Silvermines depression at the time of the mineralizing event (Fig. 5).

We are dealing with extreme disequilibrium fractionations of $\Delta^{34}\text{S}_{\text{SO}_4-\text{H}_2\text{S}}$ between 37 and 60‰ so we can say that the bacterial reduction of sulphate provided the major source of sulphur for the tubes. Although sulphate-reducing bacterial remains have not been isolated from the deposit, indirect evidence for their probable presence may be indicated by the notably high total organic carbon content of type I pyrite, which gave a value of 8.6% (total organic carbon analysis by LECO Carbon Analyser). The bacterial reduction of sulphate has been discussed at considerable length, both experimentally (see refs 28–30) and theoretically (see ref. 31), elsewhere. Despite the thorough nature of such research, detailed interpretation of bacterially produced sulphur isotope results has not been possible for ancient environments, where precise details of the physical, chemical and particularly bacterial (/organic) environments have been obliterated by time.

In our model (Fig. 5) we envisage the most significant bacterial reduction of sulphate taking place in the halocline (the gradational salinity interface between 'normal' seawater and the brine pool). The salinity in this region could be low enough not to inhibit bacterial activity; and suspended carbonaceous material, accumulated in a density trap, would provide an adequate reservoir of organic material necessary for the reduction process. Normal seawater would not host these bacteria since they are obligate anaerobes. Present-day stratified basins indicate organic and bacterial concentration in the halocline^{32,33}, thus corroborating our model.

Chimneys and rods have only been found in the higher parts of the Silvermines basin, specifically adjacent to the main east-west fault. We suggest that this was in the region at which the halocline intersected the sea floor.

This region may also provide the more oxidizing conditions required for pyrite precipitation, for example, by way of the notional reaction (at pH ~7.5):



We suspect also that the type II structures formed nearer the base of the halocline than type I. This is suggested from the fact that type I chimneys were completely absent from the Upper 'G' deposit which is structurally lower than the Ballynoe deposit, where both types are found.

To explain the differences in fractionation between type I and type II tubes we invoke variations in their formation site and/or their formation time (with respect to the mineralizing event).

For example, it has been noted that the larger the sulphate pool, the greater the expected degree of fractionation³². Thus, because of the more elevated sites of type I vents with respect to the brine pool, it is likely that sulphate availability would be greater there than at type II sites, where sulphate depletion through baryte precipitation would have occurred. Additionally, the sulphate reservoir available for reduction in the centre of type I pipes is likely to have been less than at the periphery, and this may account for the isotopic zonation exhibited by these vents (see Fig. 3).

An attractive interpretation of the sulphur isotope results hinges on the time factor. If we consider that the extent of fractionation resulting from the bacterial reduction was $\Delta^{34}\text{S}_{\text{SO}_4-\text{H}_2\text{S}} = 40\%$ throughout economic deposition at Silvermines, then approximately -20‰ (a value corresponding to the deposit average) would be the $\delta^{34}\text{S}$ expected from reduction of Lower Carboniferous seawater sulphate^{26,34}. This relates directly to type II vent results. Type I vents, however, would need a considerably lighter sulphate ($\delta^{34}\text{S} \approx 0\%$) for reduction to produce fractionation up to -40‰. Such a light sulphate would accrue if bacterially produced 'normal' sulphides ($\delta^{34}\text{S} \approx -20\%$) were oxidized to sulphate in the brine pool (a process involving insignificant fractionation, hence resultant sulphate $\delta^{34}\text{S} \approx -20\%$) and subsequently homogenized with seawater sulphate. The mixing ratio of these two reservoirs was unlikely

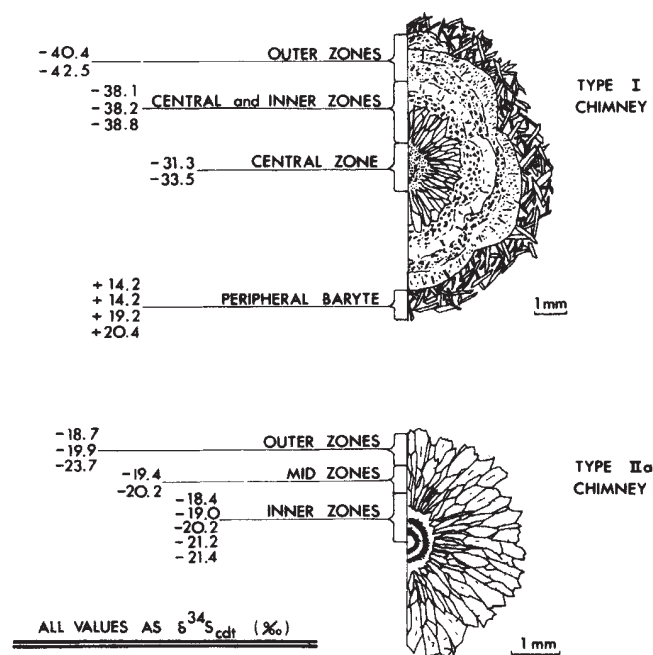


Fig. 3 Zonal representation of S-isotope results (as $\delta^{34}\text{S}_{\text{CDT}}\text{‰}$) from types I and IIa hydrothermal chimneys from Silvermines. All results are from analyses of pyrite unless otherwise indicated.

to have been constant, hence the 10% spread in $\delta^{34}\text{S}$ ratios exhibited by the type I chimneys and rods.

Temporally the latter circumstance would relate to the waning of a mineralizing pulse or to periods of quiescence. Subsequent introduction of metals would precipitate the unusually light sulphide. This then, may have been the period of precipitation for type I vents, whereas type II vents precipitated during active mineralization, when most sulphide would have been precipitated and thus have been unavailable for oxidation. Perhaps then, considering this model, it is no coincidence that the lightest reported sulphide $\delta^{34}\text{S}$ from any Lower Carboniferous, Irish orebody occurs adjacent to the lightest reported sulphate $\delta^{34}\text{S}$ from these deposits. However, because of the probable localized and transient nature of these circumstances, we would not expect a significant light sulphate reservoir to have existed at Silvermines.

A third alternative is that type II vents are a hybrid of epigenetic sulphide ($\delta^{34}\text{S} \approx +3\text{‰}$) and bacterially produced sulphide ($\delta^{34}\text{S} \approx -30\text{‰}$ to -40‰). Such an assumption is consistent with the probable greater hydrostatic pressure implied by the structurally deeper position of most type II tubes with respect to palaeo-sea level which we may expect to have hindered volatilization of primary H_2S .

The peripheral baryte of the chimneys exhibits a range of 6.2% (from +14.2 to +20.4%) and the limited data presented have a bimodal distribution. Given an average Lower Carboniferous seawater value of around +19.5% (refs 6, 26, 34) it is evident that $\delta^{34}\text{S}$ was depleted in some of this baryte. We consider an isotopic contribution from minor oxidation of sulphide to be responsible for this ^{34}S depletion. Such an explanation was previously suggested for a similar variation in the S-isotope ratios of sulphate in the Red Sea brine, which spanned 5.9% (from +16.0% to +21.9%)³⁵. We suspect, however, that the tendency of peripheral baryte ratios to exhibit bimodality is probably a facet of the small number of analyses.

A vadose origin for the tubular pyritic structures is ruled out by the discovery of these extreme fractionations of the sulphur

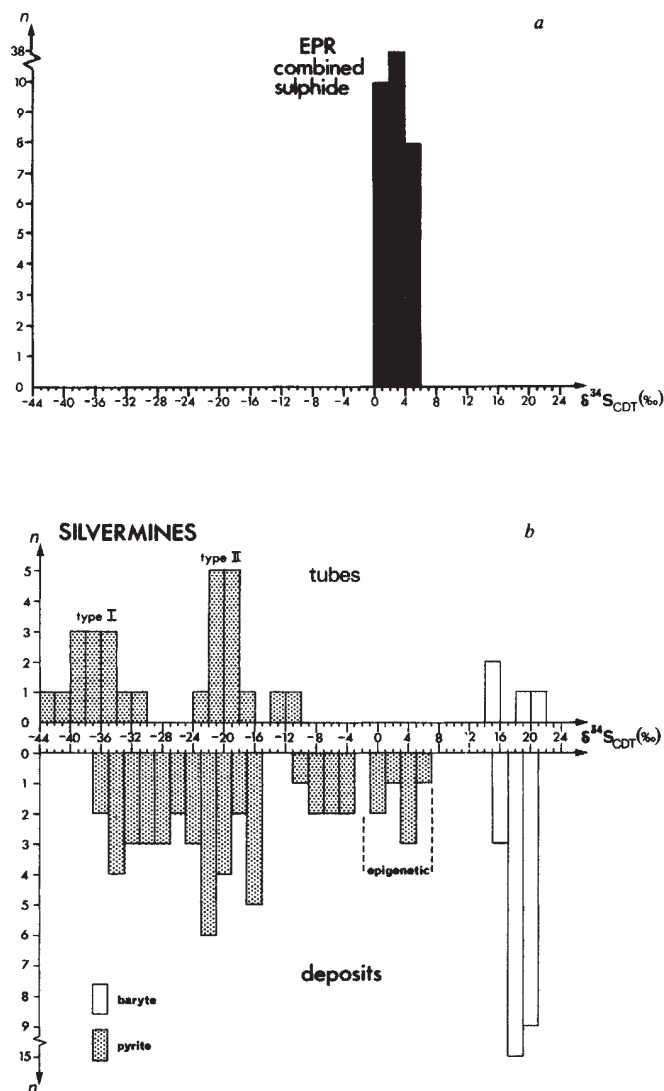


Fig. 4 a, Histogram of all published S-isotope data from the sulphides of the East Pacific Rise hydrothermal sites^{10,11,38}. b, Histogram comparing the S-isotope results of the Silvermines tubes with the published results of the same minerals from the associated deposits^{6,22}.

isotopes in the sulphides. Where true sulphide stalactites do occur, at Pine Point in Canada, their $\delta^{34}\text{S}$ values are indistinguishable from those in the hypogene ores which have a tightly defined average of +20.1% ($\sigma = 2.6\%$) (refs 36, 37, and J. R. Kyle and D. F. Sangster, personal communication).

Relevance for mineral exploration

Our study shows that S-isotope analyses could be a valuable tool in intra-prospect exploration, even where the interpretation of the results is quite subjective. The mounds and chimneys mark the main areas where upwelling mineralizing solutions approach towards, and discharge into, the sea. Larter *et al.*⁴ pointed out that epigenetic sulphide and sulphate developments may be sited below these zones, hence their utilization as pointers to mineralization is potentially valuable (particularly considering that the type IIb vents were found above the Pb + Ag-rich Lower 'G' zone on the same segment of the Silvermines fault). Even where chimney structures are destroyed by

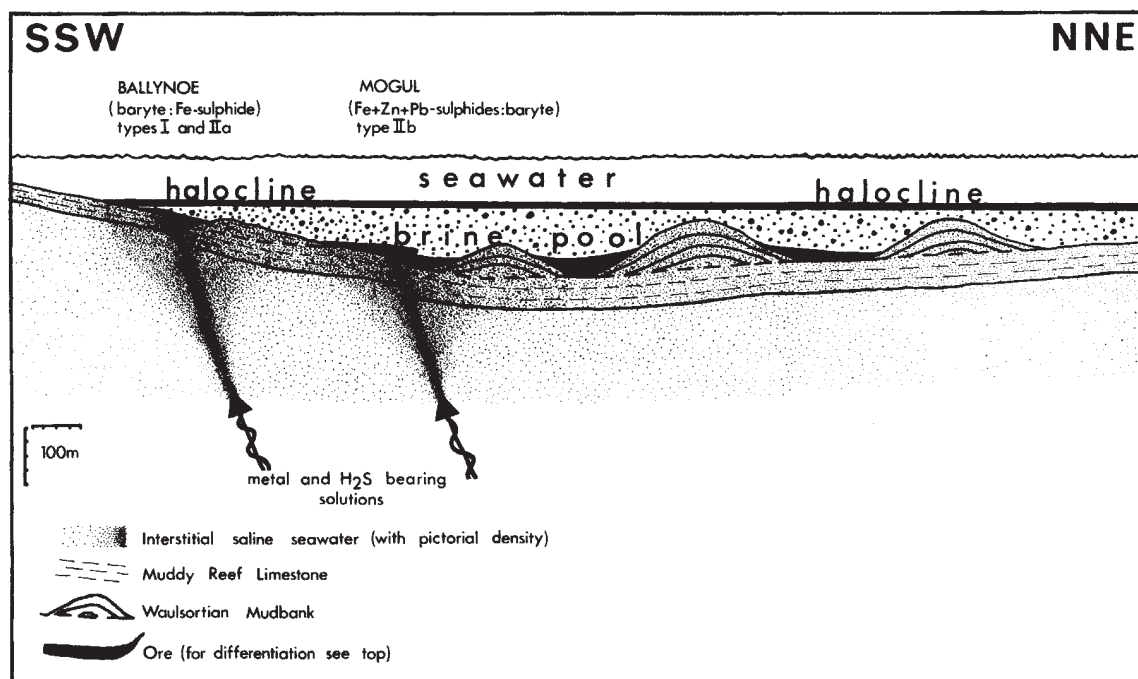


Fig. 5 Model depicting the environment of mineral precipitation at Silvermines. Metal-rich exhalant carries $\Sigma^{34}\text{S}$ sulphide at +3‰ (primary) in fluid of ~12 wt% NaCl equiv. Interstitial seawater and brine pool have salinity ~20 wt% NaCl equiv. The halocline hosts SO_4 reducing bacteria, and organic material which has been caught in a saline density trap. Bacterial fractionation produces sulphide $\delta^{34}\text{S}$ values between -18.4‰ and -42.5‰ in the tubes.

metamorphism, or remain undiscovered, a sulphur isotope study on this type of sediment-hosted exhalative deposit (formed within a brine pool), may be of practical value in revealing feeder mound sites, since unusually light $\delta^{34}\text{S}$ results seem to be diagnostic of such material.

We expect similar isotopic variations, particularly the very light sulphide sulphur values around the fossil hydrothermal vents, and the primary (heavy) sulphide sulphur of the lower feeder zones, to be revealed by comprehensive studies on orebodies like Navan in Ireland, and H. Y. C. McArthur River and Lady Loretta in Australia. Moreover, present day chimneys may yet be discovered exhibiting similar fractionations where

hydrothermal fluids issue in a halocline above a brine pool developing in a constricted ocean such as the Red Sea.

We thank the staff and workers of Magcobar and Mogul Mines for patient and willing assistance, particularly Graham Smith and Stewart Taylor. We thank with Dic Larter, Iain Samson, Allan Hall, Paul Duller, Andrew Russell, John Sherwood and Rob Willan for valuable discussions. The invaluable help of Marg Cox and John Rouse during S-isotope analyses is gratefully acknowledged. Chris Cornford (Britoil) kindly ran the T.O.C. analyses. A.J.B. is supported by a NERC award. M.L.C. publishes with the approval of the Director, Institute of Geological Sciences (NERC).

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Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development

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Nucleotide sequence and deletion analysis have been used to identify the regulatory and coding sequences comprising the cholera toxin operon (ctx). Incorporation of defined in vitro-generated ctx deletion mutations into Vibrio cholerae by in vivo genetic recombination produced strains which have practical value in cholera vaccine development.

MODERN history has recorded seven world pandemics of cholera, a diarrhoeal disease produced by the Gram-negative bacterium *Vibrio cholerae*¹. Laboratory tests can distinguish two biotypes of *V. cholerae*, classical and El Tor, the latter being responsible for the most recent cholera pandemic. The diarrhoeal syndrome induced by colonization of the human small bowel by either biotype of *V. cholerae* is caused by the action of cholera toxin, a heat-labile enterotoxin secreted by the growing vibrios². Diarrhoeal diseases affecting both humans and animals caused by some enterotoxigenic strains of *Escherichia coli* are also induced by a heat-labile enterotoxin (LT) which is closely related to cholera toxin in structure and mode of action¹⁻³.

Cholera toxin is an 84,000-molecular weight (MW) protein composed of one A subunit (27,000 MW) and five B subunits (11,600 MW). The A subunit, although synthesized as a single polypeptide chain, is usually proteolytically nicked to form two disulphide-linked polypeptides, A1 (22,000 MW) and A2 (5,000 MW)^{4,5}. The A1 polypeptide is an enzyme and promotes the activation of adenylate cyclase in target cells by catalysing the ADP-ribosylation of a GTP-binding regulatory component of the cyclase complex⁶. The resulting accumulation of cyclic AMP in the intestinal mucosa leads to the severe fluid loss characteristic of cholera. Each B subunit has a high binding affinity for the toxin's cell surface receptor, ganglioside GM₁ (ref. 7). Neutralizing antibodies raised against the holotoxin react mainly with the B subunits^{1,2}.

Much of the current interest in the genetics of cholera toxin has been promoted by the need to develop a more efficacious vaccine against this enterotoxic disease. Parenterally administered, killed whole-cell and toxoid vaccines have been shown to be largely ineffective in producing long-lasting immunity to cholera, presumably because they lack the ability to induce local immune responses in the intestine^{8,9}. Since the natural disease is capable of inducing prolonged immunity^{9,10}, several investigators have proposed the use of attenuated, non-toxinogenic mutants of *V. cholerae* as live oral cholera vaccines¹¹⁻¹⁶. While encouraging results in volunteer studies have been obtained with some of these strains, factors such as genetic instability or poor colonizing ability have contraindicated their use in the field¹⁵⁻¹⁷.

The recent relaxation of US governmental guidelines prohibiting the molecular cloning of bacterial toxin genes has permitted the use of a powerful new approach to the analysis of cholera toxin gene structure and vaccine development. These studies have shown that like the *elt* genes, which encode *E. coli* LT¹⁸, the genes for the A and B subunits of cholera toxin are arranged in a single transcriptional unit with the A cistron (*ctxA*) preceding the B cistron (*ctxB*)¹⁹. *V. cholerae* strains of the classical biotype contain a nontandem, chromosomal duplication of the *ctx* operon that is structurally identical in all strains.⁵³ In contrast, about 70% of El Tor strains have only a single copy of

ctx, while the remaining strains have two or more *ctx* copies present on a tandemly repeated genetic element. This genetic duplication and amplification of the toxin operon may be related to the instability observed in some of the earlier *V. cholerae* toxin mutants^{13,16}.

In this article, we report the entire nucleotide sequence of one *ctx* operon together with partial sequences containing the *ctx* promoter regions of five other cloned *ctx* copies. Deletion analysis has allowed the identification of toxin transcriptional and translational regulatory sequences. An *in vitro*-constructed, internal deletion in *ctxA* was recombined *in vivo* into both *ctxA* gene copies of *V. cholerae* strain Ogawa 395. Since this genetic recombinant still produces the immunogenic B subunit of the toxin, it should have practical value in cholera vaccine development.

Molecular cloning of *ctx* operon copies

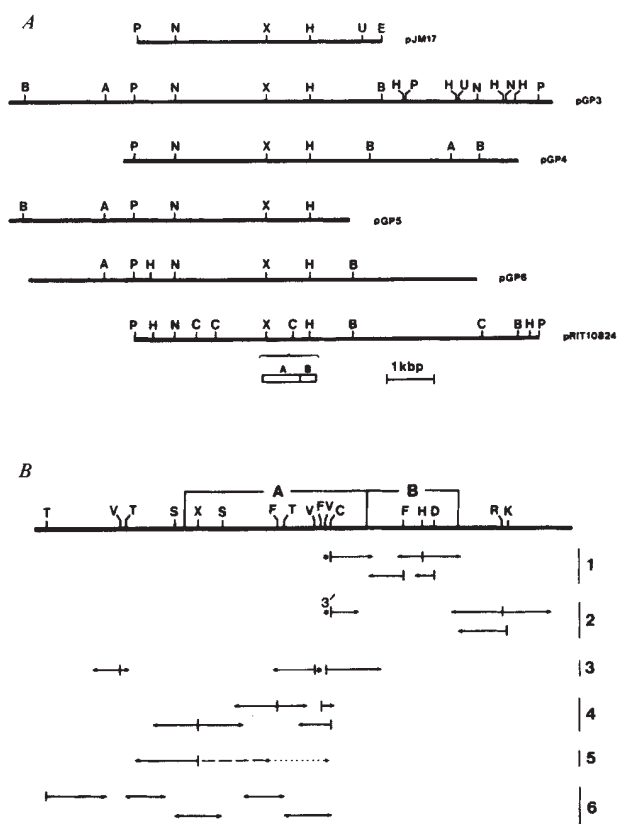
A total of six *ctx* copies were cloned from four *V. cholerae* strains. These include both *ctx* copies from strain 569B, both copies from strain RV79, one of two copies from strain E7946 and the single *ctx* copy of strain 2125. With the exception of the classical strain 569B, all these strains are El Tor in biotype. The *ctx* copies were cloned as various *V. cholerae* restriction fragments which hybridized with ³²P-labelled *elt* or *ctx* probes as described in the legend to Fig. 1.

The restriction sites for several endonucleases were located on these cloned inserts, and the resulting maps were aligned at the conserved *Xba*I site previously determined to lie early in the A cistron¹⁹, (Fig. 1A). Other conserved restriction sites for *Nru*I, *Pst*I, *Ava*I and *Bgl*II were also found preceding *ctxA* on these various inserts (Fig. 1). Additional restriction mapping and hybridization analysis has indicated that the 5 kilobase pairs (kbp) of DNA directly preceding the toxin structural genes is the same for all cloned *ctx* copies thus far examined⁵³. Since the larger chromosomal sequence environment flanking these different *ctx* copies appears to vary as determined by Southern blot hybridization, we have proposed that the conserved DNA immediately upstream of *ctx* is part of a genetic element responsible for toxin operon duplication and transposition events. From a practical point of view, the conserved 5' flanking sequences associated with *ctx* provided part of the necessary homology for efficient *in vivo* recombination of *in vitro* constructed deletion mutations into multiple copies of the toxin operon (see below).

Nucleotide sequence of *ctx*

The strategy used to determine the complete nucleotide sequence of the single *ctx* copy of strain 2,125 is shown below the restriction map of the insert cloned on pRIT10824 (Fig. 1B). The 2,020 nucleotides determined are shown in Fig. 2. Comparison of the 2,125 nucleotide sequence with both the *elt* nucleotide sequence^{20,21} and known amino acid sequences of

Fig. 1 A, Restriction maps of cloned *V. cholerae* DNA fragments carrying *ctx* genes. The maps are aligned over the common *Xba*I restriction site in the A subunit coding sequence. The limits of the coding regions for the A and B subunits are shown below the maps while the names of plasmids carrying the various inserts are shown to the right. pJM17 and pGP3 each contain, respectively, the two different *ctx* copies from strain 569B. pGP4 and pGP5 contain the two different *ctx* copies from strain RV79, and pGP6 contains one of two *ctx* copies from strain E7946. pRIT10824 contains the single *ctx* copy cloned from strain 2125. **B**, Enlarged map and sequencing strategy used to determine the sequence of the 2125 *ctx* operon. The upper line shows an expanded restriction map covering the junction between the 1.6 kbp and 3.9 kbp *Cla*I fragments on pRIT10824. The location of the A and B subunit coding regions is indicated above the line, while the arrows below denote the sites, direction and extent of sequencing. The following letters are used to denote different restriction enzyme sites on the maps; A, *Ava*I; B, *Bgl*II; C, *Cla*I; D, *Dde*I; E, *Eco*RI; F, *Hinf*I; H, *Hinc*II; K, *Hae*III; N, *Nru*I; R, *Rsa*I; S, *Sau*3A; T, *Taq*I; U, *Pvu*I; V, *Hpa*II; X, *Xba*I. **Methods:** Cloning methods. The construction of plasmid pJM17¹⁹ involved the cloning of an *elt*-homologous *V. cholerae* *Pst*I-*Eco*RI restriction fragment recovered by electroelution from agarose gel slices. A similar strategy was used to clone the single *ctx* copy from strain 2125 into pBR322⁴¹ as three independent fragments: a 1.6 kbp *Cla*I fragment (in pRIT10841) hybridizing to an *eltA* gene probe, a 3.9 kbp *Cla*I fragment (in pRIT10810) hybridizing to an *eltB* gene probe, and an 8.3 kbp *Pst*I fragment (in pRIT10824) hybridizing to the 1.6 kbp *Cla*I fragment of pRIT10841. The *eltA* and *eltB* gene probes and hybridization conditions were those previously described⁴². Only the map for the 8.3 kbp insert of pRIT10824 is shown here. The other *ctx* inserts cloned on plasmids pGP3, pGP4, pGP5 and pGP6 were isolated from genomic libraries of strains 569B, RV79 and E7946 prepared by insertion of *Sau*3A-partially digested chromosomal fragments into the *Bam*HI site of plasmid pBR327⁴¹ (see below). Only partial restriction maps of the inserts present on pGP3 and pGP5 are shown in this figure. **Construction of genomic libraries:** About 250 µg of *V. cholerae* DNA was partially digested with *Sau*3A (New England Biolabs) by incubation in the presence of 33 units of enzyme at 37 °C for 10 min in a buffer containing 6 mM Tris-HCl, pH 7.5, 5 mM MgCl₂ and 50 mM NaCl, followed by heat inactivation at 70 °C for 10 min. The digested DNA was fractionated by velocity sedimentation through a 10–40% sucrose gradient, and fractions containing 7–12 kbp fragments were precipitated by addition of two volumes ethanol. Approximately 1 µg of this partially digested DNA was mixed with 2 µg of pBR327⁴¹ which had been previously digested with *Bam*HI (New England Biolabs) and bacterial alkaline phosphatase (Bethesda Research Laboratories). The DNA mixture was incubated at 16 °C for 6 h, in 300 µl of a solution containing 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 0.4 mM ATP, 5 mM dithiothreitol, and 600 units of T₄ DNA ligase. Transformation of this ligation mixture into *E. coli* MS371 (K-12 F⁻ gal⁺ thi⁺ endA⁺ sbcB⁺ hsdR4⁺ hsdM⁺) yielded a library consisting of 0.5–2 × 10⁴ ampicillin resistant transformants, of which 90% were tetracycline sensitive and carried recombinant plasmids with 7–11 kbp *V. cholerae* DNA inserts. Libraries were screened by colony hybridization using ³²P-labelled *eltA* and *eltB* gene probes and hybridization conditions as described previously^{19,42}. **DNA sequencing:** Maxam and Gilbert sequencing⁴³ was done on the following plasmids: (1) pRIT10810, (2) pRIT10809, same as pRIT10810 but with its 3.9 kbp *Cla*I insert in the reverse orientation, (3) pRIT10824 or (4) pRIT10841. The sequencing was performed on 5'-labelled fragments or in the one case indicated a 3'-labelled fragment from pJM17, pGP3, pGP4, pGP5 and pGP6 as follows: the dotted line denotes the extent of sequencing done on 3'-labelled fragments derived from pJM17, the dashed line for fragments from pJM17 and pGP3, and the solid line for fragments from plasmids pJM17, pGP3, pGP4, pGP5 and pGP6. An asterisk denotes that labelling was at an adjacent site on the plasmid vector. Polynucleotide kinase and the Klenow fragment of DNA polymerase I were used, respectively to label the 5' and 3' ends of fragments essentially as described elsewhere³⁶. Sequencing using M13³⁶ was done by the dideoxy method⁴⁴ after subcloning *Taq*I or *Sau*3A fragments from pRIT10824 or pRIT10841 into the M13mp7 vector³⁷. A synthetic, phage-specific primer was used to initiate complementary strand synthesis.



569B derived cholera toxin subunits^{22–26}, shows that nucleotides 516 to 1,292 and 1,289 to 1,663 form the coding sequences for *ctxA* and *ctxB*, respectively. The primary translation product of *ctxA* is a 258 amino acid long polypeptide, while that of *ctxB* is a 124 amino acid polypeptide. As in the case of LT^{20,21}, both polypeptides are apparently precursors with 18 and 21 amino acid hydrophobic, amino-terminal signal sequences. The A2 coding sequence lies at the carboxy-terminal end of the A subunit. The calculated molecular weights of the mature subunits are 21,817 for A1, 5,398 for A2, and 11,677 for B.

Comparison of the DNA-deduced 2,125 protein sequences, with polypeptide sequences^{22–26} determined for the 569B toxin subunits, revealed some major differences. Since partial sequencing of the two 569B *ctxA* copies showed no changes with respect to the 2,125 sequence from nucleotide 413 to 830 for the pGP3 *ctxA* copy and from 413 to 1,194 for the pJM17 copy, the discrepancies found in this region are most likely mistakes in amino acid sequencing. The agreement with the A2 polypeptide sequence is good except for an Ile-Asp inversion (residues 229–230) and an Asp-Asn transition (residue 238). The five differences between the deduced polypeptide sequence of the B subunit of 2,125 toxin and the sequence determined for the corresponding 569B polypeptide^{25,26} could be the result of single base changes.

Comparison of *elt* and *ctx* revealed that at the nucleotide level, A and B cistrons of the two different toxin operons are

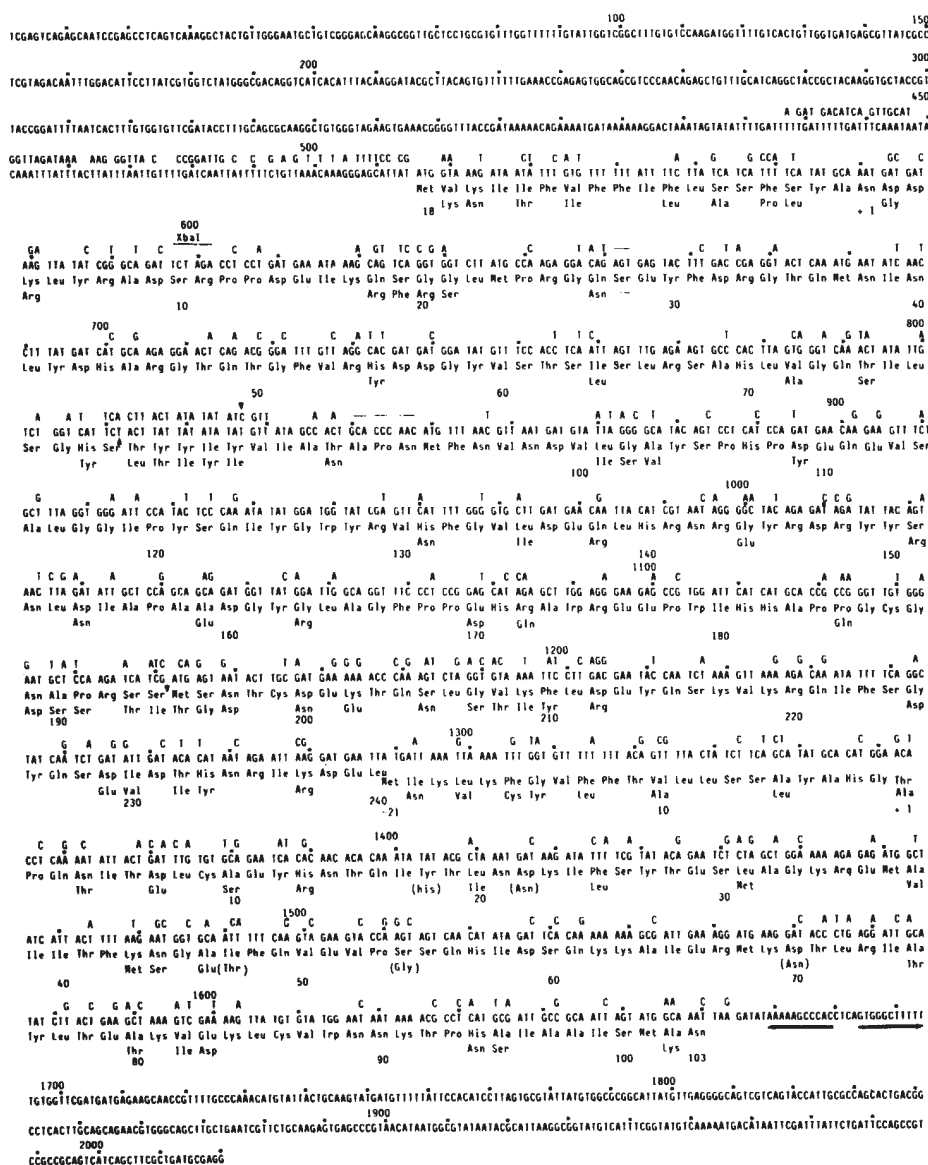
75% and 77% homologous. This percentage is reflected at the amino acid level (76% and 78% homology for A and B polypeptides, respectively). The amino acid sequences have been largely conserved, with the differences scattered throughout the sequence except for the region around the cleavage site between A1 and A2 where the homology drops to 33% between amino acids 189 and 212. Also, the A subunit of LT is four amino acids shorter than the cholera toxin A subunit.

With respect to noncoding regions, only 90 nucleotides upstream of the *eltA* initiation codon are available for comparison. In marked contrast to the coding sequences, the *elt* and *ctx* operons have diverged completely in these potential promoter regions. With regard to transcriptional termination, nucleotides 1,669–1,694 of the *ctx* sequence exhibit dyad symmetry (Fig. 2) with a G+C-rich stem-loop and a poly(T) tail depending on the length of the stem. These features are characteristic of prokaryotic, ρ -independent transcription termination signals^{27,28} and suggest that *ctxA* and *ctxB* are the only genes in the *ctx* operon.

Translational signals in *ctx* expression

The *ctxA* gene has two overlapping Shine-Dalgarno (SD) sequences²⁹ of which GGAG is the more correctly spaced, relative to the ATG start of the structural gene (Fig. 3). The *ctxB* gene exhibits a theoretically perfect SD sequence (TAAGGA) which lies within the carboxy terminal coding

Fig. 2 DNA sequence of the *V. cholerae* toxin operon from strain 2125. The antisense strand is shown from 5' to 3'. From nucleotide 427 to 1,663, the sequence is compared with the published sequence of LT genes^{20,21}; *elt* nucleotides are shown above the sequence only where they differ from the *ctx* sequence except between nucleotides 810 and 830, where deletion of a T (arrowed) in the *elt* sequence creates a frameshift which is corrected by insertion of a C (arrowed) 16 bp downstream (see text). Analogous events have previously been seen after pseudoreversion of frameshift mutations⁴⁵ but to our knowledge, this is the first naturally occurring case described. The deduced amino acid sequence of *ctxA* (nucleotide 516 to 1,289) and *ctxB* (nucleotide 1,289 to 1,660) is shown and compared with that of LT. Amino acids that differ in LT are shown below the cholera toxin amino acid sequence. In addition, for the mature B subunit sequence, differences from the published amino acid sequence of B subunit purified from strain 569B^{26,27} are shown in brackets. Two of these differences (amino acids 47 and 54) are also found in LT. The cleavage site between the A1 and A2 polypeptides is indicated by an arrow (amino acids 194–195). Note also the overlap of *ctxA* and *ctxB* cistrons (nucleotides 1,289–1,292). Sequence exhibiting dyad symmetry and potentially involved in transcription termination is indicated with divergent arrows. Features of the sequence immediately upstream of the *ctxA* gene are detailed in Fig. 3.



sequence (nucleotides 1,277–1,282, Fig. 2) of the *ctxA* cistron. The first two nucleotides of the *ctxA* translation termination signal TGA are the last two nucleotides of the *ctxB* translation initiation triplet ATG. This particular overlapping arrangement is also found several times in phage λ operons³⁰ and may be involved in translational coupling³¹ of the *ctxA* and *ctxB* genes. However, evidence presented below suggests that this is not the case with the *ctx* operon. Where documented, translational coupling is observed between cistrons whose gene products interact in a one to one stoichiometry³¹, and in contrast, the cholera toxin molecule is composed of one A subunit and five B subunits. Moreover, *E. coli* produces stoichiometrically 7 times more cholera toxin B subunit than A subunit (data not shown). Fusion of the *ctxB* gene to various *E. coli* promoters allows high expression of *ctxB* in the absence of *ctxA* translational initiation signals. These data suggest that translation of *ctxB* relies primarily on independent initiations promoted by its own ribosome binding site.

Another experiment supports this conclusion. Our DNA sequencing analysis identified two *NdeI* sites at positions 561 and 1,337 within the *ctxA* and *ctxB* genes, respectively. The positions of these sites relative to the reading frames of *ctxA* and *ctxB* allowed us to construct a *ctxA* deletion which codes for an in-frame fusion of amino acid 17 of the A subunit signal sequence to amino acid 19 of the B signal and thus maintains the normal processing site of the B signal sequence (residue

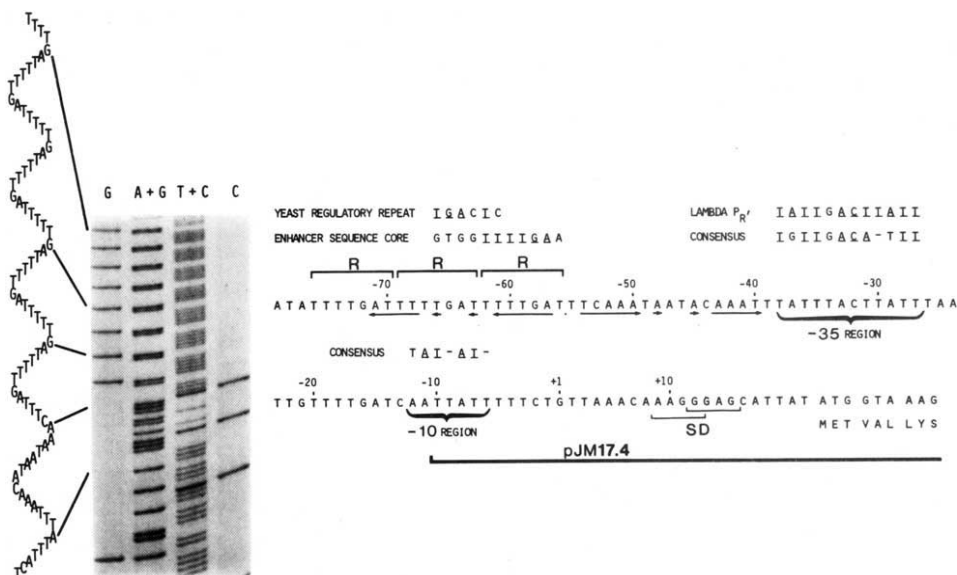
21). This genetic fusion makes B subunit expression dependent on the efficiency of the A cistron translation initiation sequences, provided the hybrid signal sequence is processed at normal efficiency. *NdeI* digestion of plasmid pGP3 followed by ligation produced such a fusion between these two sites and gave plasmid pJM3.1. Plasmid pJM3.1 produced $0.056 \mu\text{g ml}^{-1}$ of B subunit in *E. coli* MS371 while pGP3 produced $0.50 \mu\text{g ml}^{-1}$. These data suggest that the *ctxB* ribosome binding site is about ninefold more efficient than the *ctxA* site.

Toxin promoter regions

We determined approximately 200 base pairs of sequence upstream of the *XbaI* sites for each of the other five additional cloned copies of the *ctxA* gene, cloned on plasmids pGP3, pGP4, pGP5, pGP6 and JM17. Comparison of these sequences with the corresponding region of the *ctxA* gene derived from strain 2,125 indicated a perfect conservation of sequence between these copies from nucleotides 413 to 590 with one notable exception. The sequence TTTTGAT comprising nucleotides 419–425, 426–432 and 433–439 of the 2,125 sequence was found tandemly repeated 3–8 times preceding different *ctxA* gene copies (Fig. 3). Figure 3 shows part of a sequencing gel autoradiograph that spans DNA carrying eight of these tandem repeats in the region adjacent to the *ctxA* gene of pJM17.

To determine the position of the toxin operon promoter with respect to these repeated sequences, we used nuclease *Bal31*

Fig. 3 DNA sequence preceding the start of the *ctxA* cistron. The sequence of the antisense strand of DNA corresponding to nucleotides 416 to 524 of the 2125 sequence is shown. The sequence is numbered relative to the proposed +1 mRNA start point at nucleotide 495. Sequences constituting the -10 and -35 regions of the proposed toxin promoter are indicated. Above these are consensus sequences determined by Pribnow⁴⁶ and Rosenberg and Court²⁷ to be present, with some variation, in corresponding regions of most *E. coli* and virus promoters. The -35 region of the λ P_R promoter³² is also shown for comparison. Nucleotides underlined in the consensus and P_R sequences are also present in the proposed *ctx* promoter sequence. SD refers to nucleotides which are complementary to sequences located near the 3' terminus of 16S rRNA²⁹. The DNA removed (+ or - two nucleotides) from this region by the deletion carried by plasmid pJM17.4 is indicated by the lower heavy line. Divergent arrows under the indicated nucleotides designate regions of hyphenated dyad symmetry. Three copies of the tandemly repeated sequence TTTTGAT are indicated (R). This sequence was found tandemly repeated at this site three times for the *ctxA* genes from strains 2125 and RV79, four times for one of the two *ctxA* copies from strain E7946 and eight times for each of the *ctxA* copies from strain 569B. The figure also shows that this repetitive region exhibits nucleotide sequence homology with a repetitive element present in Ig and viral enhancer sequences⁴⁷ and the core sequence of a yeast regulatory repeat⁴⁸. To the left of the nucleotide sequence is part of a sequencing gel autoradiograph showing the region 5'-proximal to the *ctxA* gene of pJM17 which carries eight copies of the TTTTGAT repeat.



to construct a set of deletions around the *Xba*I site on plasmid pJM17. Expression of the *ctxB* gene was used to score the effects of these deletions on toxin operon transcription in *E. coli* MS371 and the *V. cholerae* toxin deletion mutant M7922¹⁵. The smallest deletion upstream of the *ctxA* gene which substantially reduced B subunit production in both of these bacterial backgrounds was carried by pJM17.4. The inhibitory effect of the deletion carried by pJM17.4 was shown to be due to the small 130 bp deletion 5'-proximal to the *Xba*I site of this plasmid by analysing the expression of the reassorted plasmids pJM23, pJM31 and pJM32 (Table 1).

Figure 3 shows the sequence of the antisense strand of DNA 5'-proximal to the *ctxA* gene and the extent of DNA removed in this region by the deletion on pJM17.4. The deletion begins within *ctxA* and extends about 31 bp upstream from the start of the A cistron. Since this deletion greatly reduced expression of the B gene, it appears that some essential component of the *ctx* promoter is located within the 31 nucleotides immediately preceding the initiation codon of *ctxA*. This region contains a sequence AATTATT, which matches four of the five specific bases of the Pribnow consensus sequence, TATNATN. Given that most transcripts initiate at the first purine residue 5-7 nucleotides downstream from the Pribnow box²⁷, we have tentatively assigned the G residue located at position 495 in the 2,125 sequence as the +1 position of the toxin transcript (Fig. 3).

Our proposed -10 region is associated with a -35 region that shared 7 of 11 bases with the consensus sequence for this region²⁷ and is identical to the -35 region of the strong P_R promoter of phage λ ³² except for one G to T base change. Directly preceding the proposed -35 region of the toxin promoter lies a 32 bp region of hyphenated dyad symmetry (Fig. 3). The position of this symmetrical sequence relative to the -35 region suggests that it may have a role in positive regulation²⁷ of the toxin promoter. In regard to this possibility, we have recently cloned a *V. cholerae* regulatory gene which activates *ctx* transcription approximately 100-fold in *E. coli* (*V. Miller and J.M., manuscript in preparation*). Another characteristic of the symmetrical region is that half of its structure is contributed by DNA containing the tandemly repeated sequence TTTTGAT. It is of interest that strain 569B, which carries eight of these repeats upstream of each of its two toxin operon copies, produces about two orders of magnitude more toxin than strains

2,125, RV79 and E7946. However, the different toxin operon copies cloned from these four strains all express about the same amount of B subunit in *E. coli*. Therefore, if the repetitive region is important in determining differences in toxin expression between *V. cholerae* strains, then these sequences probably interact with regulatory components unique to *V. cholerae*. In this respect, a 30-50-fold increase in B subunit production for plasmids pJM20, pJM23 and pJM32 was observed in *V. cholerae* M7922 as compared with *E. coli* MS371 (Table 1). Since these plasmids all express B via the natural toxin promoter, and actually have copy numbers 3-4-fold lower in M7922 than in MS371 (as determined by hybridization), these data suggest that expression of the toxin operon is about 100-fold higher in *V. cholerae* than in *E. coli*.

Construction of *ctx* deletion derivatives

Ogawa 395 (0395) is a classical strain of *V. cholerae* which has been shown to induce in human volunteers protective immunity against homologous or heterologous serotypic rechallenge that was greater than 3 yr in duration^{9,10,17}. We have constructed isogenic A⁻B⁺ and A⁻B⁻ toxin derivatives of 0395 by *in vivo* recombination of *in vitro* constructed deletions of the *ctx* operon into each of the two resident *ctx* copies carried by this strain.

Plasmid pJM23 served as the starting point in these strain constructions since it carries an internal *ctxA* deletion which by DNA sequencing was shown to span nucleotides corresponding to 597 to 1,059 of the 2,125 sequence (Fig. 2). This deletion removes over 80% of the DNA sequence required for formation of the A1 polypeptide and all of the sequence corresponding to residues thought to be involved in formation of the A1 active site³³. A second *ctx* deletion was constructed which removes the DNA between the *Xba*I site at nucleotide 597 and the *Hinc*II site at nucleotide 1,515, thus deleting essentially all of *ctxA* and half of *ctxB* (Fig. 2). DNA fragments carrying the *ctx* deletions were subcloned as *Eco*RI fragments into plasmid pRK290³⁴ to give plasmids pJM290.2 and pJM290.211, respectively (Fig. 4A): The 918 bp *ctxB* deletion present on pJM290.211 is actually a deletion-substitution since a 1.4 kbp DNA fragment encoding resistance to Kanamycin (Km) was inserted at the position formally occupied by the deleted *ctx* DNA (Fig. 4A).

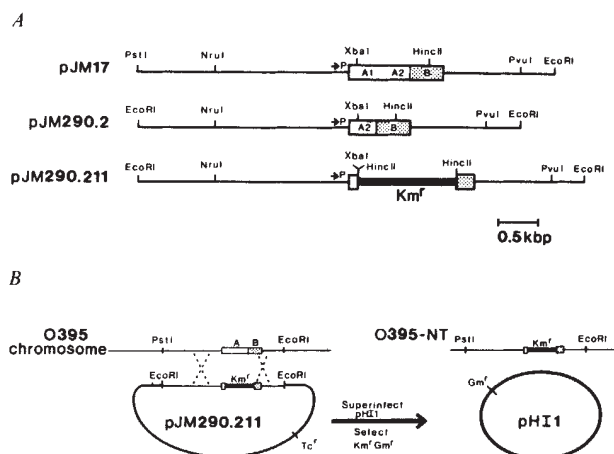


Fig. 4 A, Restriction maps of *ctx* inserts carried by pJM17, pJM290.2 and pJM290.211. B, Marker exchange procedure used to construct strain 0395-NT.

Methods: Plasmid construction. Exonuclease *Bal31* treatment of pJM17 followed by ligation in the presence of synthetic *XbaI* DNA linkers was used to construct plasmid pJM17.23 as described in Table 1. The reassorted plasmid pJM23 was constructed by replacing the 2.1 kbp *PstI*-*XbaI* fragment of pJM17.23 with the original 2.7 kbp *PstI*-*XbaI* fragment of pJM17 which carries the *ctx* promoter (arrow marked P). The *PstI* site of pJM23 was converted to blunt ends by treating 0.3 μ g of *PstI* digested pJM23 with 1 unit of T4 DNA polymerase (Bethesda Research Labs) for 10 min at 37 °C in a buffer containing 33 mM Tris-acetate, pH 8.0, 66 mM Na acetate, 10 mM Mg acetate, and 0.2 mM of each of the four deoxynucleotide triphosphates. Subsequent ligation in the presence of a 50-fold molar excess of synthetic *EcoRI* linker (Collaborative Research) followed by transformation and selection of tetracycline resistance gave plasmid pJM23.2. The 4.6 kbp *EcoRI* insert of pJM23.2 was then subcloned into the *EcoRI* site of pRK290³⁴ to give pJM290.2. A 1.4 kbp *EcoRI* fragment from pUC71K (gift from J. Vieira and J. Messing) encoding resistance to kanamycin (*Km*) and also flanked by two *HincII* sites, was modified with *XbaI* linkers and inserted in the *XbaI* site of pJM23.2 to give pJM23.21. Digestion of pJM23.21 with *HincII*, followed by ligation and selection of *Km*^r *Tc*^r transformants gave pJM23.211. The 5.6 kbp *EcoRI* insert of pJM23.211 was then subcloned into the *EcoRI* site of RK290 to give pJM290.211. The marker-exchange procedure of Ruvkun and Ausubel³⁴ was used to recombine the *in vitro*-constructed *ctx* deletion mutation carried by pJM290.211 onto the chromosome of *V. cholerae* 0395 *in vivo*. Mobilization of pJM290.211 into a spontaneous *Sm*^r derivative of 0395 was performed essentially as described in the legend to Table 1 for strain M7922 and pJM17 derivatives. Transconjugants which were *Sm*^r *Tc*^r *Km*^r carried exclusively pJM290.211 as evidenced by the nontransmissible nature of the *Km*^r and *Tc*^r markers. Superinfection with the gentamycin (*Gm*) resistance plasmid pH11 (same as pH11J)^{49,50} was performed in similar plate matings between CSH56/pH11 and 0395/pJM290.211 by selecting transconjugants on LB agar containing 30 μ g ml⁻¹ *Gm*, 30 μ g ml⁻¹ *Km* and 100 μ g ml⁻¹ *Sm*. Since pH11 and pJM290.211 are both P-group plasmids and are therefore incompatible, the *Gm*^r *Km*^r *Sm*^r *Tc*^r transconjugants obtained had recombined the *Km*^r of pJM290.211 onto the 0395 chromosome via crossover events (dashed lines) occurring between flanking homologous regions of one of the resident chromosomal *ctx*⁺ copies and the corresponding DNA on the insert of pJM290.211. Thus, the *ctx*-*Km*^r deletion-substitution mutation carried by pJM290.211 replaced the normal *ctx* locus as shown (B). However, since 0395 has two copies of the toxin locus, it was necessary to recombine the *ctx*-*Km*^r deletion-substitution mutation into the other copy as well. This was accomplished by growing cells containing one marker-exchanged copy for several generations during which time homologous sequences flanking the other *ctx* copy allowed recombination events to transfer the *ctx*-*Km*^r deletion-substitution mutation into the other *ctx* copy. 0395 derivatives carrying the *ctx*-*Km*^r deletion-substitution mutation in both *ctx* copies occurred at a frequency of 10⁻² and were recognized by colony hybridization⁵¹ using a probe (CT-1, Fig. 5) which specifically hybridizes to the DNA removed by the deletion mutation. One of these double marker-exchanged derivatives was subcultured overnight in the absence of *Gm* and underwent spontaneous curing of pH11 to give strain 0395-NT. The construction present on pJM290.2 was crossed onto the chromosome by a modified marker-exchange procedure. Plasmid pJM290.2 was mobilized into strain 0395-NT which had both of its two *ctx* copies tagged with *Km*^r. Introduction of pJM290.2 into this strain allowed cross-over events, analogous to those shown in section B, to subsequently replace both *Km*^r-tagged copies with the simple *ctxA* deletion present on pJM290.2. After overnight subculturing of 0395-NT/pJM290.2 in the absence of *Km*, these double recombinant colonies were recognized as spontaneous *Km*^r segregants, which arose at a frequency of 10⁻³ when single colonies were analysed by replica plating. One of these was spontaneously cured of pJM290.2 by subculturing in the absence of *Tc* to give strain 0395-N1. Southern blot hybridizations (Fig. 5) were used to confirm the expected structures of the 0395-NT and 0395-N1 *ctx* loci.

The marker exchange procedure of Ruvkun and Ausubel³⁴ was used to recombine the deletion-*Km*^r substitution mutation carried by pJM290.211 into one of the *V. cholerae* 0395 chromosomal *ctx* copies (Fig. 4B). Subsequent *in vivo* recombination spontaneously produced a genetic recombinant (0395-NT) that had both of its resident *ctx* copies replaced with the *ctx* deletion-*Km*^r substitution mutation carried by pJM290.211. A second *ctx* marker exchange step with pJM290.2 replaced both *Km*^r tagged *ctx* copies of 0395-NT with the internal *ctxA* deletion construction carried by pJM290.2 to give strain 0395-N1.

Southern blot hybridization, using *XbaI* genomic digests and probes derived from the *elt* and *ctx* genes, was used to confirm the genetic structures of 0395-N1 and 0395-NT. The two *ctx* loci of the parental strain 0395 were seen as two bands (9.8 and 7.9 kbp) which reacted with all three probes used in the analysis (Fig. 5). In contrast, 0395-N1 and 0395-NT were both unreactive with a probe, LT-A1, specific for the A1 region of *ctxA*. The CT-1 probe, which exactly corresponds to the *ctx* DNA deleted on pJM23.211, did not react with 0395-NT but did hybridize to two restriction fragments of 0395-N1. Each of these fragments was smaller than the corresponding 0395 band by the size of the *ctxA* deletion originally present in pJM23 (~450 bp). The CT-B1 probe, derived from DNA downstream from the *HincII* site in *ctxB*, hybridized to the same bands as the CT-1 probe for 0395-N1. The CT-B1 probe reacted with two bands in 0395-NT which were each 500 bp larger than the corresponding two bands of 0395. This is the difference in size between the 0.9 kbp *ctx* deletion carried on pJM290.211 and the 1.4 kbp *Km*^r fragment inserted at the deletion junction.

These results, together with analogous results obtained with restriction enzymes other than *XbaI*, confirmed that 0395-N1 and 0395-NT carry the appropriate *ctx* deletions mutations in both of their two *ctx* copies. Consistent with this conclusion, 0395-N1 produces the same amount of the B subunit (0.3 μ g ml⁻¹) as the 0395 parental strain but displays no toxicity detectable in Chinese hamster ovary cells³⁵, while 0395-NT produces neither the A subunit nor the B subunit of cholera toxin (data not shown).

Discussion

Our determination of the cholera toxin nucleotide sequence has confirmed the close evolutionary relationship of *ctx* and *elt*. However, the nucleotide sequence homology observed is confined entirely to the A and B polypeptide coding sequences. The 5' control regions upstream from these structural genes have completely diverged for the two toxin operons. We also found that the *ctx* operon expressed much more efficiently in *V. cholerae* than in *E. coli*. Indeed, genetic fusions of the very strong *tac* promoter³⁶ to *ctxB* actually produce only one-third as much B subunit in *V. cholerae* as the natural *ctx* promoter derived from strain 569B (J.J.M., unpublished results). These observations suggest that the structure of the *ctx* promoter region reflects its interaction with regulatory proteins unique to *V. cholerae*. The existence of a variety of *V. cholerae* toxin regulatory mutations supports this hypothesis^{37,38}. In contrast, the *elt* operon, being located extrachromosomally on plasmids², may have been forced to evolve more universal transcriptional signals which would function reasonably well in a variety of Gram-negative backgrounds.

Although we have not yet identified the function of the tandemly repeated sequence TTTTGAT found in the *ctx* promoter region, we point out that such short repetitive structures have not been previously observed in a prokaryotic organism. It is also of interest that much larger tandem duplications of regions either adjacent to or encompassing the entire *ctx* operon are also very common among strains of *V. cholerae*⁵³. Thus, *V. cholerae* may have evolved several different levels of genetic control which involve DNA amplification.

In addition to allowing the determination of the *ctx* nucleotide sequence, molecular cloning has also allowed us to construct

Table 1 Effects of *Bal31* deletions on B subunit expression

Plasmid	Size of deletion upstream of <i>Xba</i> I site	Size of deletion downstream of <i>Xba</i> I site	B subunit production	
			<i>E. coli</i> MS371	<i>V. cholerae</i> M7922
pJM17	0	0	0.3	ND
pJM17.4	0.13	0.40	<0.005	0.2
pJM32	0	0.40	0.3	10.0
pJM31	0.13	0.45	<0.005	0.2
pJM23	0	0.45	0.2	9.0
pJM20	0	0.30	0.2	10.0

Plasmid construction: five μg of pJM17 DNA¹⁹ was linearized with *Xba*I and treated with 6 units of exonuclease *Bal31* (New England Biolabs) for 2–60 min. The cut-back plasmid was phenol extracted, ethanol precipitated, and then ligated in the presence of a 50-fold molar excess of synthetic *Xba*I linker (New England Biolabs). The plasmid was redigested with *Xba*I, ligated and transformed into *E. coli* MS371 selecting for tetracycline resistance. Deletion plasmids were characterized by digesting with *Pst*I and *Xba*I to excise the fragment upstream of the original *Xba*I site of pJM17 or with *Xba*I and *Eco*RI to excise the fragment downstream of the original *Xba*I site of pJM17 (see restriction map, Fig. 1). The size of deletions in these fragments when compared to the original fragments is shown in kilobase pairs. Re-assorted plasmids (pJM20, pJM23, pJM31 and pJM32) were constructed by replacing the *Pst*I–*Xba*I or *Xba*I–*Eco*RI fragment on one deletion plasmid with the analogous fragment from other deletion plasmids (pJM17.20 or pJM17.23, not shown) or from pJM17, as indicated by the size of the deletions. Plasmids were mobilized into the *V. cholerae* toxin deletion mutant M7922¹⁵ using the plasmid pRK2013³⁴ as follows: *E. coli* MS371 carrying various pJM17 derivatives was grown in LB broth containing $15 \mu\text{g ml}^{-1}$ tetracycline (Tc) to saturation at 37 °C. About 20 μl of this culture was mixed with 20 μl of a saturated culture of MM294/RK2013³⁴. The mixtures were incubated on the surface of an LB plate at 37 °C for 3 h, and then 20 μl of a saturated LB culture of a streptomycin (Sm^r) resistant derivative of M7922 was mixed into the patch of growth. After further incubation of the plate for 3 h, M7922 transconjugants were selected on LB agar plates containing $100 \mu\text{g ml}^{-1}$ Sm and $15 \mu\text{g ml}^{-1}$ Tc. These were scored for sensitivity to $30 \mu\text{g ml}^{-1}$ kanamycin (Km) to assure that these strains did not also inherit pRK2013. Production of the cholera toxin B subunit was performed by growing cells in CYE medium³⁹ at 37 °C for 18 h with aeration in the presence of $15 \mu\text{g ml}^{-1}$ tetracycline. B subunit was measured in cell extracts of MS371 derivatives prepared as described previously¹⁹ and in culture supernatants of M7922 derivatives. As in the case of cholera toxin¹⁹, less than 5% of the total B subunit produced was found in the supernatant of MS371 derivatives and less than 1% was found in cell extracts of M7922 derivatives. The B subunit was quantitated as antigen using a GM₁-dependent, enzyme-linked immunosorbent assay⁴⁰ and purified toxin as standard. Values are reported as $\mu\text{g ml}^{-1}$ toxin-antigen equivalents, and less than $0.005 \mu\text{g ml}^{-1}$ could not be detected in the assay. ND, not determined. Due to NIH recombinant DNA guideline restrictions, plasmid pJM17 was not transferred to *V. cholerae* M7922.

specific deletion mutations in the *ctx* operon. *In vivo* recombination of these mutations into both *ctx* copies of *V. cholerae* 0395 has resulted in the construction of A⁺B⁺ (0395-N1) and A⁺B⁺ (0395-NT) derivatives of this strain. Given the fact that primary infections with strain 0395 have been shown to induce long-lasting immunity in human volunteers^{9,10,17}, we anticipate that the derivatives 0395-N1 and 0395-NT can be used as stably attenuated, live oral cholera vaccines. Previous *V. cholerae* toxin mutants developed for this purpose have been less suitable because of factors such as genetic instability of toxin mutations¹⁶ or poor colonization properties¹⁷. We have presumably avoided these problems by incorporating precise *ctx* deletion mutations into an unmutagenized parental strain with proven human colonization and immunogenic properties. Given that the parental strain 0395 carries two copies of the *ctx* operon, construction of vaccine candidates such as those described here would be essentially impossible by conventional genetic methods involving chemical mutagenesis. Thus, while it is clear that potential hazards are associated with the cloning of toxin genes, the application of this technology to the genetic manipulation of

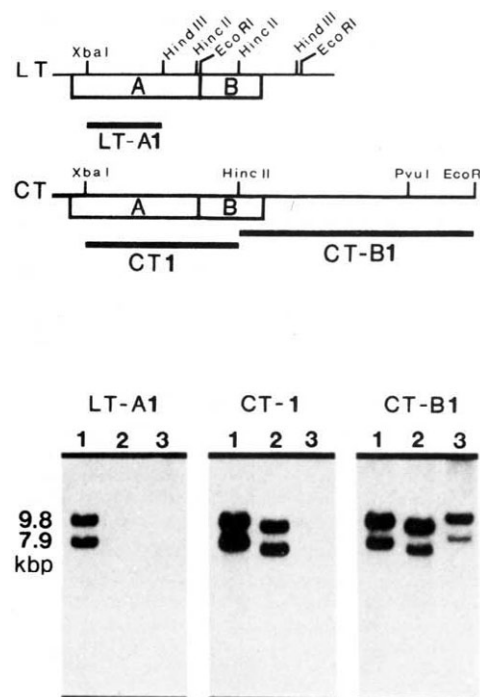


Fig. 5 Southern blot analysis of 0395 and *ctx* deletion derivatives. The top shows partial restriction maps of cloned inserts carrying the *elt* (LT) and *ctx* (CT) operons and the location of restriction enzyme fragments (heavy lines) used as probes. Shown below are exposures obtained after Southern blot analysis of DNA from wild-type and *ctx* mutant strains. The lanes contained DNA from the following *V. cholerae* strains: 1, 0395; 2, 0395-N1; 3, 0395-NT. The more efficient hybridization of the CT-B1 probe to the upper bands in all three lanes reflects the fact that this probe contains not only *ctxB* sequences but also sequences unique to the downstream flanking DNA of the *ctx* locus located on these larger *Xba*I chromosomal fragments. The sizes of the two original wild-type 0395 *Xba*I fragments containing the two *ctx* copies are shown in kilobase pairs (kbp).

Methods: Probe preparation. The LT-A1 probe is a 475 bp *Xba*I–*Hind*III fragment from plasmid EWD299¹⁸; the CT-1 probe is a 918 bp *Xba*I–*Hinc*II fragment and the CT-B1 probe is a 1.45 kbp *Hinc*II–*Eco*RI fragment, both from pJM17¹⁹. The fragments were purified by electroelution from gel slices and labelled with [α -³²P]dCTP (7,000 Ci mmol⁻¹, NEN) by nick translation³⁶. DNA from *V. cholerae* strains (1 μg) was digested with *Xba*I, fractionated by electrophoresis in 0.7% agarose gels, and transferred to a nitrocellulose sheet by the method of Southern³². The nitrocellulose was then hybridized sequentially with the LT-A1, CT-1 and CT-B1 probes. Between each successive hybridization the displayed autoradiographic exposures (lower panels) were obtained and the previous probe was removed by treatment of the nitrocellulose with 20 mM NaOH for 2 h. Hybridizations were done at 37 °C for 18 h and, depending on the probe being used, were carried out in solutions of either high (CT-1 and CT-B1 probes) or low (LT-A1 probe) stringency. High-stringency solution contained 50% (v/v) formamide, 75 mM Na citrate, pH 7.0, 0.75 M NaCl, 1 mM EDTA, 0.1 SDS, 0.05% bovine serum albumin, 0.05% polyvinyl pyrrolidone, 0.05% Ficoll and 250 $\mu\text{g ml}^{-1}$ heat-denatured calf thymus DNA; low-stringency solution was identical except that it contained 25% (v/v) formamide.

toxins does greatly facilitate the construction of safer and more effective bacterial vaccines.

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Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes

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DNA sequences containing the 5'-flanking regions of the insulin and chymotrypsin genes were linked to the coding sequence of the chloramphenicol acetyltransferase (CAT) gene. The insulin gene recombinant elicits preferential expression of CAT activity when introduced into cells producing insulin; similarly, the chymotrypsin gene recombinant elicits preferential expression in chymotrypsin-producing cells. Sequences located upstream of previously defined transcriptional control elements are essential for efficient expression in both cases.

DIFFERENTIATED cells in eukaryotes possess a remarkable capacity for selective expression of specific genes. A single gene may account for a large fraction of the total gene expression in one cell type, yet be expressed at undetectable levels in other cells. The level of control is very large, and may approach a million-fold^{1–3}. Current evidence suggests that the rate of transcription is a key aspect in this control of gene expression⁴. In contrast to the great specificity seen *in vivo*, it is possible to detect expression of protein coding genes of higher eukaryotes using a variety of heterologous *in vivo* and *in vitro* systems^{5–8}; however, the levels of expression observed are extremely low compared with those found in differentiated cells *in vivo*. Analyses of these activities have revealed the presence of a number of distinct transcriptional control elements located close to the mRNA initiation site. One of these sequences (the TATA or Goldberg-Hogness box) is located about 30 base pairs (bp) upstream from the initiation site. The TATA sequence is present in most, but not all, genes and helps to determine the efficiency and the precise location of the transcription start site⁹. Two less well defined elements are located further upstream: the first occurs 70–80 bp from the transcription start site, and has the consensus sequence CCAAT^{10,11}, and the second lies 80–110 bp upstream from the transcription start site^{11,12}. Careful analyses of the expression of the β -globin gene and the herpes thymidine kinase (TK) gene have failed to reveal a functional role for DNA sequences further upstream than around 110 bases^{11,12}. No evidence exists linking these elements to cell-specific expression.

Study of viral transcription units has identified a different class of control element typically located 100–300 bp upstream

from the transcription start site. These elements, termed enhancers, are distinguished by their ability to activate transcription units independently of their orientation or precise positioning relative to the unit^{13–16}. Enhancers operate in a variety of cell types, although not necessarily with equal efficiency: the polyoma enhancer, for example, is functional in differentiated mouse cells but not in undifferentiated embryonal cells^{17,18}.

There have been relatively few reports documenting introduction of genes into differentiated cells which express the endogenous cognate gene^{19–24}. Stable transformants of myeloma cells express higher levels of a transferred immunoglobulin κ gene than similar transformants of fibroblasts²². Expression of the chick δ crystallin gene is higher on microinjection into lens cells than fibroblasts²⁴.

We have designed experiments to test for elements of genes required for cell-specific expression. These involve linking a putative regulatory control region to an enzymatic 'reporter' function²⁵. The genes coding for mammalian insulin^{26–29} and rat chymotrypsin B (G. I. Bell, C. Quinto, C. S. Craik and W.J.R., unpublished results) have been cloned and characterized. They are expressed at high level only in the pancreas. However, clearly distinct cell types are involved: insulin is synthesized in endocrine β -cells and chymotrypsin in exocrine cells³⁰. We linked DNA sequences containing the 5'-flanking region of the insulin and chymotrypsin genes to the coding sequence of chloramphenicol acetyltransferase (CAT), a particularly useful reporter function, developed by Gorman *et al.*³¹. Insulin and chymotrypsin gene recombinants were introduced into pancreatic endocrine and exocrine cells as well as into non-pancreatic cells, and the relative levels of transient

expression of the CAT gene measured. The use of an enzymatic reporter function combined with transient expression procedures provide an effective and facile method for detecting and characterizing cell-specific gene expression.

Construction and testing

To facilitate subsequent construction of 5' deletions, the *Hind*III-*Bam*HI fragment from pSVO CAT³¹ was inserted into pBR322 to generate pBR CAT (Fig. 1). DNA fragments containing the 5'-upstream regions of the following genes were inserted to pBR CAT at the *Hind*III site using *Hind*III linkers: (1) rat insulin 1²⁶, (2) human insulin²⁸, (3) rat chymotrypsin *B*, (4) rat growth hormone³², (5) TK¹². These constructs were designated according to the inserted fragment; for example, the construct with the rat insulin 1 gene fragment is pBR·rIns·CAT.

To test for preferential expression of CAT recombinants following transfection to appropriate cells we used the following cell lines: (1) HIT cells, a transformed line from hamster pancreatic endocrine cells³³ that produces insulin at 2–5% of the rate of endocrine β -cells. (2) AR4-2J cells, a rat exocrine pancreas tumour line³⁴ that contains about 10% of the level of chymotrypsin mRNA of the adult rat pancreas (A.B., unpublished). (3) CHO cells, a fibroblast line derived from Chinese hamster ovary.

Table 1 Activity of DNA containing 5'-flanking sequences

Fragment	CHO	HIT	AR4-2J
RSV	100	100	100
TK	15	14	17
Rat insulin	1.2	71	<0.4
Human insulin	<0.1	4	ND
Rat chymotrypsin	0.9	0.2	42
Rat growth hormone	<0.1	<0.2	ND

Relative CAT activities directed by DNA containing 5'-flanking sequences. For each cell type, activity is expressed as a percentage of that directed by the RSV fragment. Construction of plasmids is described in Fig. 1 legend. Activities were determined as described in Fig. 2 legend. Results shown represent the mean of two independent determinations. The range of activities around a particular value varied by up to 30% of that value. ND, not determined.

Cell-specific expression

Tissue culture cells vary widely in their ability to take up and express exogenous DNA³⁵. For example, when the Rous sarcoma virus (RSV) promoter was used to activate the expression of CAT sequences (pRSV CAT)³⁶, the CAT activity observed in HIT and AR4-2J cells relative to that in CHO cells was 30% and 1%, respectively. However, a similar ratio of activity of the

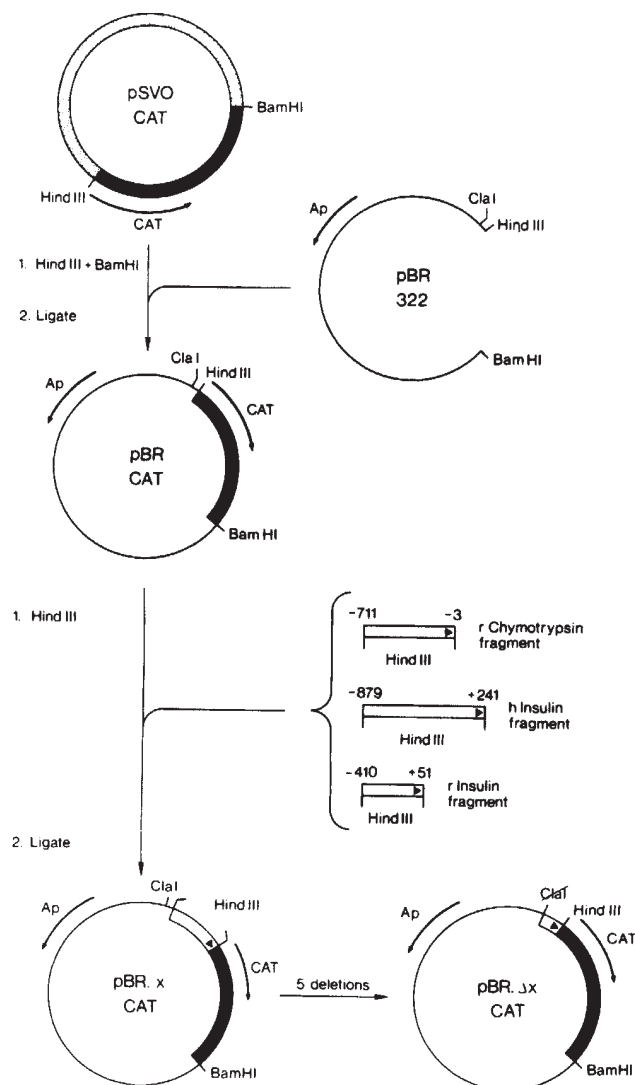


Fig. 1 Construction of recombinants containing 5' upstream fragments of insulin and chymotrypsin genes linked to the CAT coding sequence³¹. A 1.6-kb *Hind*III-*Bam*HI fragment including the entire CAT coding sequence, and also containing simian virus 40 (SV40) splice sites and polyadenylation signals, was purified by agarose gel electrophoresis. This fragment was introduced into pBR322 by ligation at the *Hind*III-*Bam*HI sites. Fragments of the 5'-flanking DNA of the rat and human insulin genes and the rat chymotrypsin gene were introduced into pBR CAT using *Hind*III linkers to generate the plasmids collectively indicated as pBR·x·CAT in the figure. The numbers at the border of the fragment define its position in base pairs relative to the transcription start site. The following fragments were used: (i) Rat insulin 1 gene, a *Pvu*II-*Rsa*I fragment comprising 410 bp of flanking DNA, 43 bp of exon 1 and 6 bp of intron 1. (ii) Human insulin gene, an *Nco*I-*Nco*I fragment comprising 879 bp of flanking DNA, 42 bp of exon 1, 179 bp of intron 1 and 20 bp of exon 2. (iii) Rat chymotrypsin *B* gene, an *Eco*RI-*Hind*III fragment comprising 709 bp of upstream flanking DNA (from -711 to -3). (iv) Rat growth hormone gene, an *Eco*RI-*Xho*I fragment comprising 1.6 kb of flanking DNA and 8 bp of exon 1. (v) TK gene, a *Bam*HI-*Bgl*II fragment comprising 109 bp of flanking DNA and 41 bp of the single TK exon. In the case of RSV, the construct used was pRSV CAT³⁶, which contains 524 bp of the RSV 3'-long terminal repeat located at the *Hind*III site of pSVO CAT. Plasmids containing deletions in the 5'-flanking DNA were constructed using two alternative procedures: in cases where appropriate restriction sites are present within the eukaryotic fragment, that site was cleaved by the restriction enzyme and ligated to the unique *Cla*I site located nearby in the pBR322 sequence, producing the plasmid pBR·Δx·CAT. When appropriate sites were unavailable, sequences were deleted by digesting with exonuclease III⁵² (exoIII). In order that the end points of exonuclease III-produced shortened fragments be joined to the identical plasmid sequences (the *Cla*I site), an intermediate plasmid containing additional sequences at the *Cla*I site was used as substrate for exonuclease III. These additional sequences comprised a 548-bp fragment of plasmid DNA with a unique *Bgl*II site located 5 bp from one end. When the intermediate plasmid was linearized with *Bgl*II and treated successively with exonuclease III, S₁ nuclease, *Cla*I, DNA polymerase I (Klenow fragment) and T4 DNA ligase, the appropriate deletions were produced. The ends of these shortened fragments were defined by DNA sequence analysis. During these constructions, the *Cla*I recognition sequence was lost as a consequence of the use of *Cla*I followed by ligation. For all constructions, plasmid DNA was isolated using standard recombinant DNA procedures⁵³ including two cycles of purification by centrifugation in CsCl gradients.

TK promoter to the RSV promoter was seen for the different cells (Table 1). This supports the view that they represent essentially 'neutral' promoters with respect to the specificity of their expression. These promoters were therefore used to control for the variability of different cell types in uptake and expression of exogenously added DNA. In marked contrast to the RSV and TK promoters, the DNA sequences containing the 5'-flanking regions of the rat and human insulin genes and the rat chymotrypsin gene exhibited very different ratios of activities in the related differentiated cells from those in other cells (Fig. 2, Table 1): both the rat and human insulin gene fragments were 50–200-fold more active in HIT cells than in either CHO or AR4-2J cells. The activity of the rat insulin gene fragment was also very low in COS 7 cells (SV40 transformed monkey kidney cells³⁷) and Alexander cells (human hepatoma cells³⁸) (data not shown). Thus, efficient CAT expression directed by the rat insulin gene fragment was only observed in insulin-producing cells. In an analogous fashion, the chymotrypsin gene 5'-flanking region showed 50–200-fold higher activity in AR4-2J cells than in either CHO or HIT cells (Table 1) or in the rat fibroblast line, Rat 2 (data not shown).

To test whether preferential expression in our system is due to selective stabilization or replication of plasmid DNA, we introduced the human insulin gene fragment into pBR CAT containing the TK promoter (pBR-TK-CAT) at a position about 2 kilobases (kb) downstream from the 3' end of the CAT gene. This plasmid had identical activity to the construction without the insulin gene fragment, in both CHO and HIT cells (data not shown). If selective stabilization or replication were involved, we would have expected this plasmid to be more active in HIT cells than CHO cells.

Sequences required for efficient expression

To determine the location of functionally significant sequences in the 5'-flanking regions, deletions of the 5' ends of the insulin and chymotrypsin genes were produced. The new ends were joined to exactly the same sequence of plasmid DNA (the *Clal* site). The deletion of analogous regions of the 5'-flanking sequences of these genes led to a dramatic reduction in activity in the appropriate differentiated cells (Fig. 3). The 5' border of the active sequence of the rat insulin gene fragment was located within the 56-bp region between -247 and -302 bp from the transcription start site: the border for the human insulin gene fragment was between -168 and -258 bp and for the rat chymotrypsin gene fragment between -192 and -274 bp. This active region has several interesting features: (1) It corresponds roughly to the location of a DNase I hypersensitive site estimated to extend to 250–300 bp upstream from the transcription start site of the rat insulin II gene in insulin-producing tumour cells³⁹. (2) The rat insulin I gene²⁶ contains an alternating purine/pyrimidine tract (TGTGCTGTG) located at -256 bp, similar to sequences which have been associated with Z-DNA and viral enhancers⁴⁰. (3) The sequences of rat and human insulin gene 5'-flanking regions are 75% homologous up to -240 bp²⁸. This homology disappears further upstream except for two short sequences. One of these (GTGGAAA), located at -310 bp, matches the consensus sequence proposed for viral enhancer elements⁴¹: this sequence was not required for optimal activity in our system (see Fig. 3). In addition, the polymorphic region of the human insulin gene⁴², located upstream of -354 bp, did not influence expression in our system (Fig. 3). It

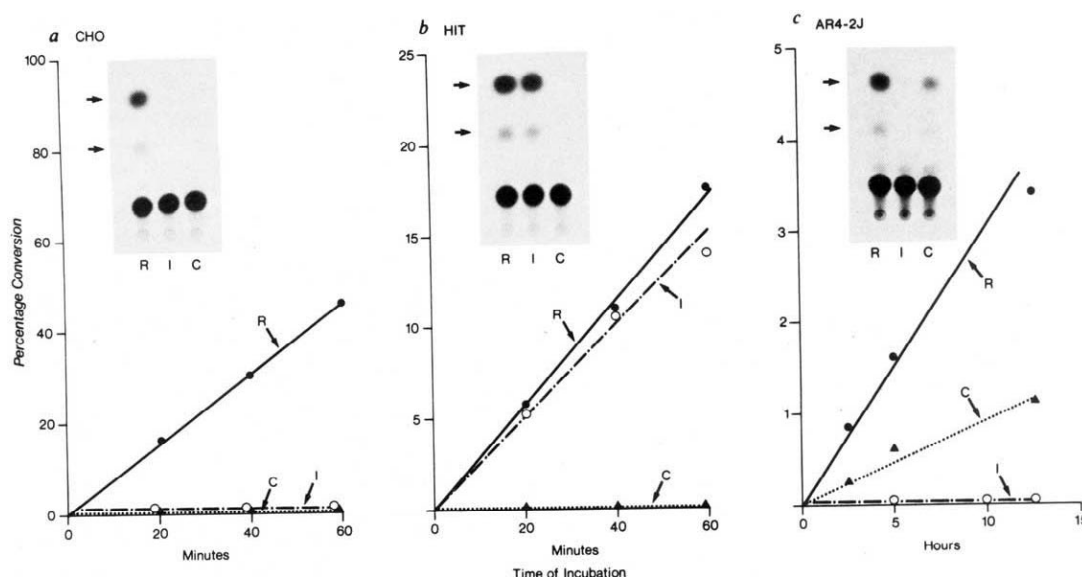


Fig. 2 CAT activity directed by eukaryotic 5'-flanking sequences in different cells. Recombinant plasmids containing the 5'-flanking regions of RSV, abbreviated R (●), rat insulin, abbreviated I (○), and rat chymotrypsin, abbreviated C (▲), were linked to the CAT coding sequence (see Fig. 1 legend). A calcium phosphate co-precipitate³⁴ containing 10 µg of DNA was added to CHO cells (a), HIT cells, subclone T15 (b) and AR4-2J cells (c) in 20–30% confluent 100-mm dishes. Four hours after the addition of the DNA, cells were subjected to 20% glycerol for 2 min. Cells were collected 44 h after addition of the DNA, and extracts prepared by sonication and centrifugation³¹. CAT assays³¹ contained in a final volume of 180 µl: Tris pH 7.5 140 mM, acetyl coenzyme A 0.44 mM, ¹⁴C-chloramphenicol (40–60 mCi mmol⁻¹, NEN) 0.2 µCi, and cell extract containing 25 µg of protein or less. Maximal reaction rates were achieved with this concentration of ¹⁴C-chloramphenicol. The reactions were allowed to proceed for up to 60 min (CHO and HIT cells) or 12.5 h (AR4-2J). In the longer reactions, a higher initial concentration of acetyl coenzyme A (4.4 mM) was necessary to preserve the linearity of the assay. Samples were extracted with 1 ml ethyl acetate, the solution was dried down and the residue redissolved in 20 µl ethyl acetate and analysed by ascending TLC using chloroform/methanol (95:5 v/v). X-ray film was exposed by the chromatograms and activities quantitated by counting regions of the chromatograms in a liquid scintillation spectrometer. Results are expressed as percentage conversion of ¹⁴C-chloramphenicol to ¹⁴C-chloramphenicol acetate by extract containing 25 µg of protein. The insets show typical autoradiograms of CAT assays. The upper two spots, indicated by arrows, correspond to the two forms of ¹⁴C-chloramphenicol monoacetate while the lower intense spot corresponds to unreacted ¹⁴C-chloramphenicol. The reaction times used were 20 min (a), 60 min (b) and 12.5 h (c).

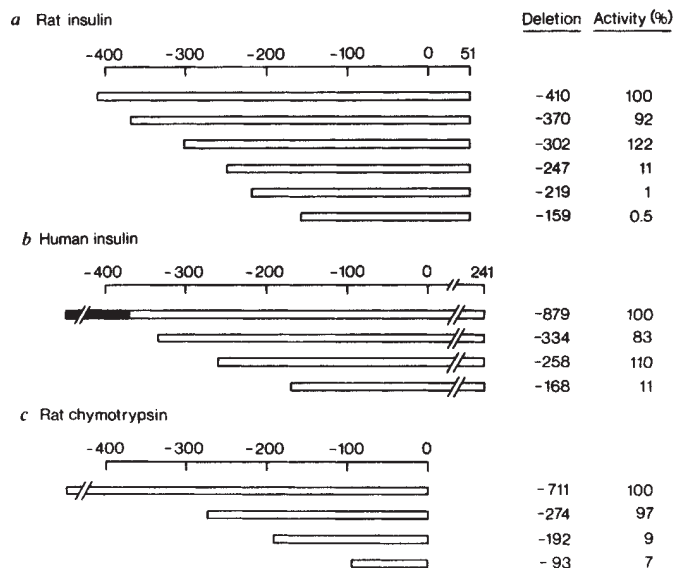


Fig. 3 Relative activity of insulin and chymotrypsin gene fragments containing 5' deletions. Deletions from the 5' end of the above fragments were produced as described in Fig. 1 legend. Some of the deletions in the gene fragments were produced by restriction enzymes: *Xmn*I (-370 and -247 in the rat insulin gene), *Pst*I (-159 in the rat insulin gene), *Pst*I (-334 in the human insulin gene), *Pvu*II (-258 in the human insulin gene), *Bgl*II (-168 in the human insulin gene), *Sac*I (-274 and -93 in the rat chymotrypsin gene) and *Nco*I (-192 in the rat chymotrypsin gene). Other deletions were produced by *exo*III digestion (-302 and -219 in the rat insulin gene). The precise end points of these latter deletions were determined by DNA sequencing. The end points of all shortened fragments are indicated in base pairs relative to the transcription start site. Activities were determined following transfection of appropriate cells (HIT cells for CAT plasmids containing insulin gene sequences and AR4-2J cells for CAT plasmids containing chymotrypsin gene sequences). CAT activities were determined as described in Fig. 2 legend. Assay times used were 60 min (CHO and HIT cells) and 12.5 h (AR4-2J cells). The relative activities are expressed as a percentage of the activity of the largest fragment tested for each gene. The location of the polymorphic region of the human insulin gene is indicated by the shaded bar.

has been postulated that variation in the length of this region influences the incidence of various forms of diabetes^{43,44}.

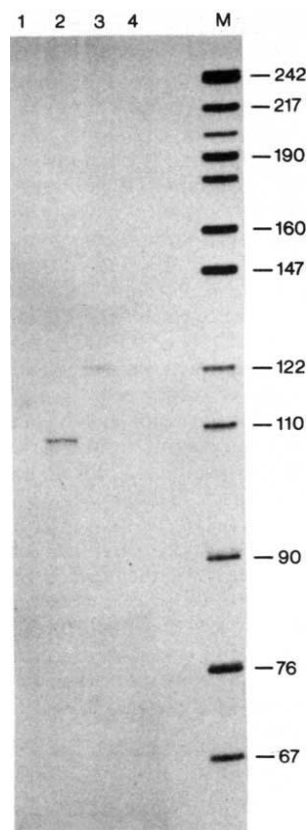
Transcription initiation

To determine whether expression of the recombinants was under the control of the expected promoters, we analysed the CAT transcripts in transfected cells by primer extension. Total RNA from HIT cells transfected with pRSV CAT produced a major product of ~107 bp in length (Fig. 4, lane 2), corresponding to the length predicted for utilization of the RSV promoter⁴⁵. When pBR·rIns·CAT was used the major extended product was ~120 bp in length (Fig. 4, lane 3); this corresponds to the predicted length of a transcript initiated under the direction of the authentic rat insulin promoter²⁸. The 120-bp band was 45% as intense as the 107-bp band, as determined by densitometric scanning of the autoradiogram. On deletion of sequences upstream from -247 bp of the rat insulin gene, the 120-bp band was barely visible on the original autoradiogram at 15% of the level obtained using the intact pBR·rIns·CAT (Fig. 4, lane 4). When CHO cells were transfected with pBR·rIns·CAT, no CAT transcripts were detected (data not shown). Thus, the relative intensity of the bands produced by primer extension corresponded to the relative activities of 5'-flanking regions as measured by CAT assay, confirming that CAT activity measurements reflect steady-state levels of CAT transcripts³⁶.

It is difficult rigorously to exclude differential mRNA stability as being a contributory factor to the results. However, differen-

Fig. 4 Analysis by primer extension of CAT transcripts in transfected HIT cells. The autoradiogram shown presents data from primer extension using RNA prepared from HIT cells transfected with the following: no DNA, lane 1; pRSV CAT, lane 2; pBR·rIns·CAT, lane 3; pBR·rIns·CAT deleted beyond -247 bp (see Fig. 3 legend), lane 4. The lane marked M displays the size (in nucleotides) of radioactive fragments produced by *Msp*I digestion of pBR322 and treatment with DNA polymerase I (Klenow fragment) in the presence of [α -³²P]dCTP. The predicted hybrid transcripts contain 71 nucleotides of CAT sequences and either 36 nucleotides of RSV sequences (combined length 107) or 49 nucleotides of rat insulin gene sequences (combined length 120).

Methods: Twenty-four hours after exposure of HIT cells to DNA, RNA was isolated by guanidinium thiocyanate extraction followed by centrifugation through a gradient of CsCl⁵⁵. A single-stranded oligonucleotide of sequence 5' CAACG GTGGT ATATC CAGTG 3', synthesized by chemical methods, was provided by Dr P. J. Barr. This oligonucleotide is complementary to nucleotides 15-34 of the coding sequence of the CAT gene⁵⁶. It was end-labelled using [γ -³²P]ATP (7,000 Ci mmol⁻¹ ICN) and T4 polynucleotide kinase (NEN) and 10 ng (specific activity 2 × 10⁷ c.p.m. pmol⁻¹) was mixed with 30 µg of RNA from transfected HIT cells in a volume of 3 µl, and incubated for 5 min at 65 °C. To this was added 1 µl of a solution containing: 500 mM Tris-HCl pH 8.3, 500 mM KCl, 80 mM MgCl₂, 400 mM dithiothreitol. The mixture was cooled to 42 °C. Thirty-two units of reverse transcriptase (Life Science) and dATP, dCTP, dGTP and dTTP (final concentration of each, 1 mM) were added to a final volume of 10 µl. Following 30 min incubation at 42 °C, the mixture was treated with phenol/chloroform and analysed by electrophoresis on an 8% polyacrylamide urea gel, followed by autoradiography.



tial mRNA stability cannot account for the loss of activity following deletion of sequences at the 5' end of the gene fragments, since identical transcripts are involved. We therefore believe it is likely that our results reflect different rates of initiation of transcription.

Discussion

We have used gene transfer techniques to probe the mechanisms involved in cell-specific gene expression. Cell-specific expression was reconstituted in the presence of DNA sequences containing 5'-flanking DNA of the insulin and chymotrypsin genes. The experiments have delineated the approximate 5' boundary of a previously unidentified control element, at least part of which is located upstream of the transcriptional control elements defined for other genes of higher eukaryotes. Further, the location of the 5' boundary of this element is similar in the genes studied.

As the fragment of the rat chymotrypsin gene used contained no transcribed sequences, the cell specificity observed in this case is associated only with flanking DNA. The rat and human insulin gene fragments, on the other hand, contain exon sequences and the human insulin gene fragment contains, in addition, a complete intron. However, in preliminary experiments, when all DNA from the transcribed portion of the human insulin gene fragment was deleted (3' end point of shortened fragment = -20 bp from the transcription start site), the full CAT-

promoting activity of the original fragment was retained. It is therefore likely that the 5'-flanking sequences are solely responsible for the cell-specific expression observed in these experiments.

As the exogenously added DNA is almost certainly not incorporated at the chromosomal locus of the endogenous gene⁴⁶, the native chromosomal environment is not an absolute prerequisite for cell-specific expression. Further, as the transcriptionally competent DNA is probably episomal in the conditions of these experiments, it seems likely that integration into chromosomes is also not essential.

This artificial system, involving transient expression of an exogenous gene, does not mimic precisely the expression of insulin and chymotrypsin by differentiated cells in two significant aspects. (1) In contrast to the stringency of cell-specific expression *in vivo*, there was detectable expression following introduction of gene fragments into control cells. The gratuitous low level activity of genes following introduction into heterologous cells has been reported in many systems⁵⁻⁷. In some cases the activity has been shown to be associated with use of abnormal promoters^{47,48}. The activity may also reflect the absence of constraints on gene expression imposed by the natural gene environment. (2) The expression of RSV RNA in infected mammalian cells (0.1% of mRNA)⁴⁹ is considerably lower than that of insulin mRNA in endocrine β -cells (20%). However, the rat insulin gene and RSV flanking regions elicited approximately equivalent CAT activities in transfected HIT cells (Table 1). The lower activities of the 5'-flanking fragments may be related to the depressed levels of differentiated function (HIT cells, 2-5% of normal; AR4-2J cells, 10% of normal) in the cells used. Thus, factors required for high level expression of the exogenous genes may be present in sub-optimal amounts. Further, in these experiments, it is possible that only a subpopulation of the transfected cells exhibit selective expression. For these reasons, we believe that our experiments underestimate the true magnitude of the regulatory control exerted through this locus.

The upstream regulatory element is clearly required for expression in appropriate differentiated cells. We have attempted to define whether it affects activity in other cells: since the low levels of activity directed by the rat insulin and rat chymotrypsin gene fragments did not diminish when we tested the series of 5'-deleted fragments in CHO cells (data not shown), the collective results suggest that the mapped element

is involved in determining cell-specific expression. The position of the 5' border of the mapped element roughly corresponds to a DNase I hypersensitive site³⁹. It is possible that the element is involved in the process of generating an open chromatin structure^{50,51}. Alternatively, it may function more directly in the process of transcription. Preliminary experiments indicate that the element can augment the activity of promoters in an orientation-independent manner, and may therefore increase transcription by mechanisms related to those used by viral enhancer elements.

The loss of activity associated with progressive deletions at the 5' end of the flanking region suggests that the element may be part of a positive regulatory system. We propose, as the simplest interpretation of our results, that a *trans*-acting positive regulatory factor, which we term a differentiator, interacts with the upstream control element; this results in selective gene expression in the differentiated cell. In principle, a relatively small number of differentiators may be sufficient to direct the many alterations in gene expression characteristic of the differentiated state of a particular cell type.

The present work provides a system for exploration of the details of this phenomenon. Identification of the domains of the control sequence should allow the isolation of putative factors which interact with it, and further characterization of the regulatory process.

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Note added in proof: Following submission of this manuscript, several reports have appeared describing the presence of cell-specific control elements within introns of immunoglobulin genes⁵⁷⁻⁶⁰.

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Pure β -adrenergic receptor: the single polypeptide confers catecholamine responsiveness to adenylate cyclase

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The β -adrenergic receptor binding subunits from frog erythrocytes, hamster lung and guinea pig lung have been purified to apparent homogeneity and in all cases reside on a single polypeptide. Insertion of the pure receptors into phospholipid vesicles and subsequent fusion of these vesicles with a receptor-deficient cell conveys β -adrenergic responsiveness to the adenylate cyclase system of the acceptor cell. Such responsiveness is linearly dependent on the amount of receptor used in the fusion experiments and is independent of the receptor source. Moreover, this responsiveness displays appropriate β -adrenergic specificity. These results indicate that the β -adrenergic receptor polypeptide contains both the ligand binding site and the site responsible for mediating stimulation of adenylate cyclase activity, presumably via interaction with the guanine nucleotide regulatory protein.

A WIDE variety of biologically active molecules, including hormones, drugs and growth factors, initiate their physiological actions by binding to specific receptors located in the plasma membranes of their target cells. The interaction of these chemical messengers with their receptors is followed by highly specific responses such as regulation of enzyme activities or opening and closing of ion channels which produce characteristic changes in cellular metabolism or activity. Binding of biologically active molecules and activation of effector systems in target cells are presumably the two functions common to all receptors.

Over the past decade, the availability of ligand binding techniques has resulted in the development of methods for the purification to apparent homogeneity of several receptor binding proteins (refs 1–6, and J.L.B., R.G.L. Shorr, M.G.C. and R.J.L., in preparation). These generally appear to be integral membrane glycoproteins of molecular weight (MW) 40,000–250,000. However, despite the progress in studying the binding functions of these receptors, methodological approaches to probing their 'activating' functions have not been generally available. Thus, a fundamental question still unanswered is whether the purified receptor proteins which bind biologically active ligands contain all the necessary components for the stimulation of cellular responses, or whether additional molecular components or accessory polypeptide units might be required for transducing the binding event into a biological signal.

Among the most thoroughly studied receptor-effector systems is the β -adrenergic receptor adenylate cyclase complex which mediates the stimulatory effects of catecholamines on physiological functions in heart, smooth muscle and many other tissues. These systems are known to consist of at least three components. The first is the β -adrenergic receptor binding protein which contains the ligand binding site for catecholamine agonists. The second is a nucleotide regulatory protein (N or G/F) which binds guanine nucleotides and thereby couples agonist interactions with the receptor to the regulation of the catalytic moiety of adenylate cyclase, the third component of the system. The complete purification of the β -adrenergic binding proteins and the nucleotide regulatory proteins from several sources have been reported^{4–8}.

With the availability of pure receptor preparations, it is now possible to address mechanistic questions concerning the molecular basis of the activating functions of adenylate cyclase coupled receptors. To this end, we now report the development

of procedures that have allowed the successful insertion of pure β -adrenergic receptor binding proteins from several sources into phospholipid vesicles. These receptor-laden vesicles, after fusion, can confer adrenergic responsiveness to *Xenopus laevis* erythrocytes. These cells contain both the nucleotide regulatory protein and adenylate cyclase components but are essentially devoid of β -adrenergic receptors and thus do not normally demonstrate catecholamine-responsive adenylate cyclase activity.

Isolation

β -Adrenergic receptors coupled to adenylate cyclase exist in cell membrane preparations in extremely low concentrations (50–500 fmol per mg total protein). Thus, about 100,000-fold purification is required for the isolation of these receptors in homogeneous form. Previous work has shown that β -adrenergic receptors can be solubilized from plasma membrane preparations by the detergent digitonin, with complete retention of the ability to bind adrenergic ligands^{9,10}. This allowed the development of biospecific affinity chromatography procedures for the isolation of these receptors^{11,12}. The β -adrenergic antagonist alprenolol immobilized to Sepharose has been used successfully to purify the β -adrenergic receptor from several tissues. Combination of an affinity chromatography step coupled with size exclusion HPLC has yielded receptor preparations purified to apparent homogeneity^{4,5}.

The β -adrenergic receptor preparations chosen for the reconstitution studies reported here were isolated from frog erythrocytes, hamster lungs and guinea pig lungs because in each instance receptor purification yields a single type of subunit component of MW 58,000 for the frog⁴ and 64,000 for the lung systems (J.L.B., R.G.L. Shorr, M.G.C. and R.J.L., in preparation). Pharmacologically, these receptors have been characterized as being of the β_2 subtype. In all cases, the final elution on HPLC yields a single protein peak, as monitored by ¹²⁵I-labelling and absorbance at 280 nm, which coincides with receptor binding activity. Figure 1 shows an example of a typical profile for a purified preparation of the frog erythrocyte β -adrenergic receptor. Note that the receptor peak (fractions 17–23) is always eluted just ahead of residual digitonin (fractions >30), which accounts for the remaining absorbance at 280 nm (○) and ¹²⁵I counts (Δ) in the profile. The results of polyacrylamide slab gel electrophoresis, which will be considered in more

Table 1 Efficiency of insertion of different pure β -adrenergic receptor preparations into phospholipid vesicles

Receptor preparation	Lipids in incubation	% Efficiency of insertion	n
Frog RBC	PC (0.7 mM) +DMPC (0.3 mM)	46 $\pm$ 14	3
Guinea pig lung	PC (0.7 mM) +DMPC (0.3 mM)	27 $\pm$ 7	4
Hamster lung	PC (0.7–1.3 mM) +DMPC (0.3 mM)	26 $\pm$ 9	10

Insertion of the pure β -adrenergic receptor preparations into phospholipid vesicles was performed as follows. Receptor (1–4 pmol in 100–400 μ l of 100 mM Tris- SO_4 , 0.05–0.1% digitonin pH 7.4) was incubated with bovine serum albumin (2 mg ml^{-1}), sonicated phospholipids (at the concentrations indicated), octyl glucoside (0.8–0.9%), alprenolol (10 μ M), 90 mM NaCl and 9 mM Tris-Cl (pH 7.4) in a final volume of 0.5–0.9 ml for 20–45 min on ice. The final concentration of digitonin in these incubations was $\leq 0.04\%$. Generally, SM-2 resin (~ 0.4 g wet weight) (BioRad) was then added and the incubation mixture stirred end over end for 1 h at 4°C. The resin was removed by centrifugation for 7 min at 700–1,000 r.p.m. In some cases, the incubation mixture was eluted through an Extracti-gel (Pierce) column (0.7 cm $\times$ 2.0 cm), rather than treated with SM-2 resin. The supernatants obtained after removal of the SM-2 resin, or the eluates from the Extracti-gel columns, were incubated with polyethylene glycol (final concentration $\sim 30\%$) for 10 min at room temperature, then diluted 10–20-fold with 100 mM NaCl, 10 mM Tris-Cl (pH 7.4) and centrifuged at 250,000g for 1.5 h at 4°C. The addition of polyethylene glycol at least doubled the efficiency of insertion when the insertion was performed with soybean phosphatidylcholine (PC) and dimyristoyl phosphatidylcholine (DMPC). The resultant receptor–lipid pellets were resuspended in 100 mM NaCl and 10 mM Tris-Cl (pH 7.4) and assayed for receptor binding by elution through Sephadex G-50 columns as described previously¹⁴. n = the number of experiments in each entry. The pure β -adrenergic receptor preparations were obtained as described in Fig. 1 legend. The concentrations of PC in the incubations are expressed in terms of lipid phosphate as determined by the method of Ames and Dubin²⁷ using DMPC as a standard. The efficiency of insertion represents the ratio of ^{125}I -cyanopindolol binding in the receptor pellet to the binding by receptor before its addition to the incubation.

detail below, indicate that each preparation consists of a single type of polypeptide of MW 58,000–64,000. The specific activities of such pure β -adrenergic receptor preparations are 12,000–18,000 pmol per mg protein. These receptor polypeptides bind adrenergic ligands with a specificity identical to that obtained when binding experiments are performed with membrane or solubilized preparations¹⁰ and, as will be shown in Fig. 2 (insets), these peptides can be photoaffinity labelled with the probe ^{125}I -p-azidobenzylcarazolol.

Insertion into phospholipid vesicles

To assess the activating function of receptors it is necessary to reincorporate the purified receptor polypeptides into a lipid environment with concomitant removal of the solubilizing detergent. A major obstacle to this type of study has been the fact that only the detergent digitonin will solubilize the β -adrenergic receptor from many tissues in a form that will permit post-solubilization ligand binding and thus allow purification by the essential biospecific method of affinity chromatography. Digitonin, unlike other detergents such as the cholate derivatives and octyl glucoside, is extremely difficult to remove because of its relatively low critical micelle concentration. However, as indicated in Table 1, incubation of purified receptor preparations (in digitonin) with phospholipids and an additional detergent, octyl glucoside, followed by treatment with SM-2 resin to remove micellar detergent¹³ and then dilution in detergent-free buffer, results in the reincorporation of receptor into a particulate lipid milieu.

The pure β -adrenergic receptors isolated from the various different sources are most efficiently (25–50%) inserted into phospholipid vesicles using a mixture of soybean phosphatidylcholine (PC) and dimyristoyl phosphatidylcholine

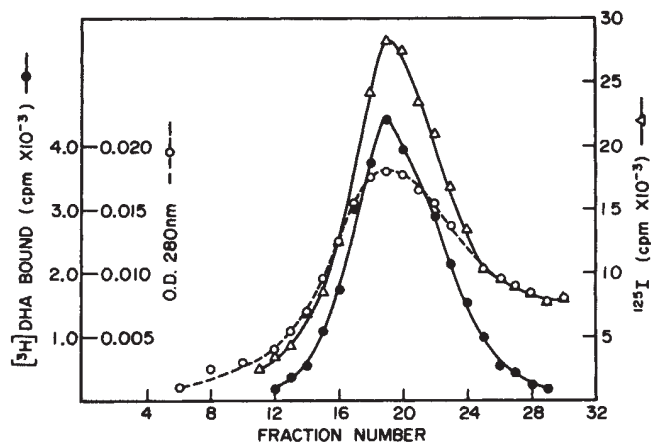


Fig. 1 HPLC elution profile of radioiodinated and unlabelled purified β -adrenergic receptor from frog erythrocytes. The fractions were monitored for absorbance at 280 nm ($\circ$), ^{125}I counts (Δ) and were assayed for ^3H -dihydroalprenolol binding ($\bullet$). The latter assays were performed on a similar HPLC run with uniodinated receptor protein.

Methods: The β -adrenergic receptor was solubilized from purified frog erythrocyte plasma membranes using 2% digitonin and purified ~ 500 -fold by affinity chromatography on Sepharose-alprenolol gel as described previously⁴ but with minor modifications. Following application of the soluble extract (300 ml) containing β -adrenergic receptor to the affinity column (200 ml) a wash of the column was performed (0°C) with 150 ml of 0.5 M NaCl, 50 mM Tris-Cl, 0.5% digitonin, pH 7.4, followed by a second wash (150 ml) with 100 mM NaCl, 10 mM Tris-Cl, 0.025% digitonin, pH 7.4. Receptor binding activity was then eluted with 100 mM NaCl, 10 mM Tris-HCl, 0.05% digitonin, pH 7.4, containing 40 μM ($\pm$) alprenolol. The partially purified receptor preparation was concentrated using an Amicon concentration cell with a YM 30 membrane, typically to a final concentration of 20–60 pmol ml^{-1} . These preparations were then purified to apparent homogeneity by HPLC (two successive passes) on steric exclusion columns (two Toyosoda TSK-4000 and one TSK-3000 tandem-linked), as described elsewhere⁴, using a mobile phase buffer of 100 mM Tris- SO_4 , 0.05–0.1% digitonin, pH 7.4. Similar procedures were used to purify β -adrenergic receptor from guinea pig lung and hamster lung (J.L.B., R. G. L. Shorr, M.G.C. and R.J.L., in preparation). The specific activities of the pure β -adrenergic receptor preparations were estimated as previously described⁴ or by the Amido Schwarz protein assays²⁸ and ranged over 12,000–18,000 pmol per mg protein. Iodinated frog erythrocyte receptor was prepared as follows: the peak of receptor binding activity after a single chromatography on the three TSK-HPLC columns was pooled. This material was iodinated by the chloramine-T method⁴, desalted on Sephadex G-50 columns to remove unreacted Na^{125}I and chromatographed on the three TSK HPLC columns as above.

(DMPC) (Table 1). Insertion into lipids can also be accomplished using mixtures of a light vesicle fraction of frog erythrocyte lipids (prepared as described previously¹⁴ and in the legend to Fig. 3) and PC, or PC alone, with efficiencies ranging over 10–50% (data not shown). Overall, these efficiencies are comparable with those obtained using less purified receptor preparations (100–5,000 pmol per mg protein)¹⁴, suggesting that the receptor binding proteins purified to apparent homogeneity contain all the necessary components for insertion into phospholipid bilayers.

Reconstitution of responsiveness

The availability of purified receptor polypeptides incorporated into phospholipid vesicles enabled us to attempt to reimplant these purified peptides into an acceptor cell, the *X. laevis* erythrocyte, by fusion procedures. This acceptor cell contains the adenylate cyclase enzyme and nucleotide binding protein but few or no β -receptors and thus no β -adrenergic responsiveness. The fusion procedures used in these studies were similar to those used previously to implant crude or partially purified receptor preparations^{14–16}.

Fig. 2 Adenylate cyclase activity in fused hybrids of different reconstituted pure β -adrenergic receptor preparations and *X. laevis* erythrocytes. Each set of data shown in the individual panels represents the means of triplicate determinations from representative experiments repeated two or three times. B, Basal; ISO, isoprenaline (50 μ M); ISO + PRO, isoprenaline (50 μ M) + propranolol (50 μ M); PGE₁, prostaglandin E₁ (3 μ M). Pure receptor preparations were inserted into vesicles containing a mixture of soybean PC and DMPC as described in Table 1 legend. The fusion of the inserted β -adrenergic receptor with *X. laevis* erythrocytes was performed using a procedure essentially identical to that developed in this laboratory previously¹⁶. Pellets containing receptor (obtained after the final centrifugation in the 'insertion' protocol) were mixed with 2×10^7 packed *X. laevis* erythrocytes. The mixture was incubated with 15 μ l of phospholipids (10 μ g lysollecithin) for 5 min and then with 10 μ l of MgCl₂ (10 mM) for 5 min. Fusion was performed with polyethylene glycol at 30 °C as previously described^{15,16,29,30}. Membranes of the resulting hybrids were prepared using freeze-thaw lysis. Adenylate cyclase assays were performed as described previously^{31,32} using 0.1 mM ATP. Assay data are reported as pmol cyclic AMP generated in a 30-min incubation period (30 °C).

Top panel: X & X, *X. laevis* erythrocytes fused with vesicles containing a mixture of soybean PC and DMPC without β -adrenergic receptor. X & β AR, 1,200 fmol of hamster lung receptor inserted into the above vesicles and fused with *Xenopus* erythrocytes. Inset: autoradiogram of SDS-PAGE of photoaffinity labelled (¹²⁵I-*p*-azidobenzylcarazolol) and iodinated purified β -adrenergic receptor used in these experiments. The left lane shows an SDS-PAGE pattern of an affinity chromatography purified sample of hamster lung β -adrenergic receptor incubated with 30 pM ¹²⁵I-*p*-azidobenzylcarazolol for 60 min at 25 °C, desalted on Sephadex G-50 and photolysed for 90 s as described previously⁵. The right lane shows the SDS-PAGE pattern of the iodinated purified receptor preparation used in these experiments. Aliquots of these samples were run on 8% SDS-PAGE. The dried gel was exposed for 24 h with Kodak XAR-5 film and developed manually. Hamster lung β -adrenergic receptor was purified essentially as described for the frog erythrocyte receptor and as will be reported elsewhere (J.L.B., R. G. L. Shorr, M.G.C. and R.J.L., in preparation). Iodination of the hamster lung β -adrenergic receptor was performed using procedures described in Fig. 1 legend for the frog erythrocyte β -adrenergic receptor except that an affinity chromatography purified sample was iodinated and subjected to HPLC. With the lung receptor preparations a single HPLC is required for purification. The arrows to the right show the relative mobility of known molecular weight standards. **Middle panel:** X & X, *X. laevis* erythrocytes fused with vesicles containing a mixture of soybean PC and DMPC without β -adrenergic receptor. X & β AR, 570 fmol of frog erythrocyte β -adrenergic receptor inserted into vesicles and fused with *Xenopus* erythrocytes. Inset: autoradiogram of SDS-PAGE of photoaffinity labelled ¹²⁵I-*p*-azidobenzylcarazolol and iodinated purified β -adrenergic receptor used in these experiments. The left lane shows an SDS-PAGE pattern of an affinity chromatography sample of frog erythrocyte β -adrenergic receptor labelled as above. The right lane shows the SDS-PAGE pattern of the iodinated purified receptor preparation used in the fusion experiments. Frog erythrocyte β -adrenergic receptor was purified and iodinated essentially as described in Fig. 1 legend. **Bottom panel:** X & X, *X. laevis* erythrocytes alone, carried through the fusion procedure. X & β AR, 500 fmol of guinea pig lung β -adrenergic receptor inserted into vesicles and fused with *Xenopus* erythrocytes. Inset: SDS-PAGE of photoaffinity labelled ¹²⁵I-*p*-azidobenzylcarazolol and iodinated purified β -adrenergic receptor used in these experiments. Purified guinea pig lung β -adrenergic receptor was prepared essentially as described for the frog erythrocyte receptor and as will be reported elsewhere (in preparation). The guinea pig lung receptor was iodinated as outlined for the hamster lung preparation above.

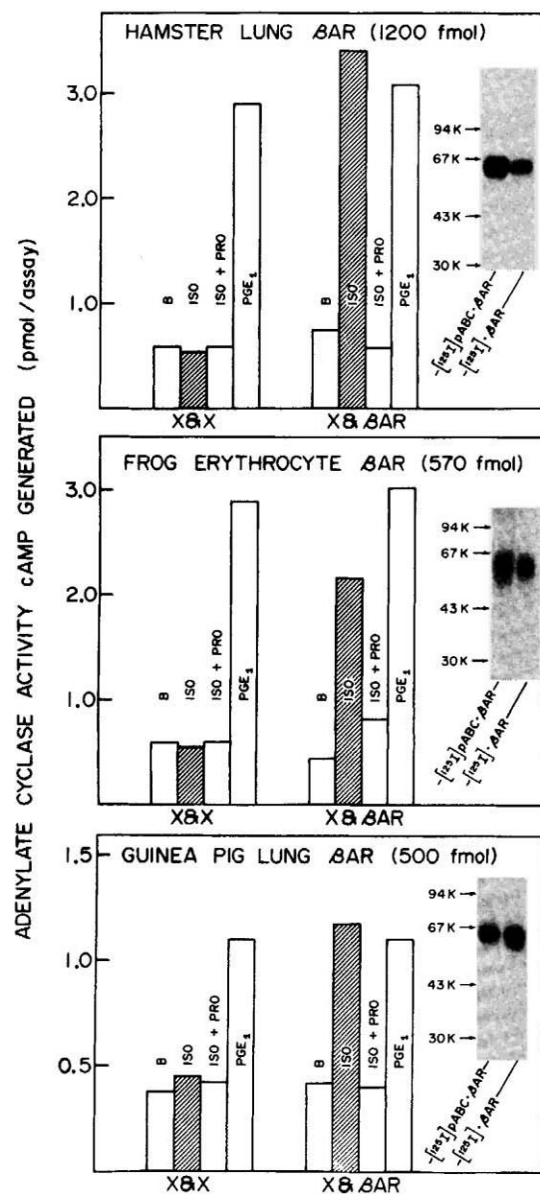


Figure 2 summarizes the results of the fusion of the different pure β -adrenergic receptor preparations inserted into phospholipid vesicles with the acceptor *X. laevis* erythrocytes. The insets to each of the panels in Fig. 2 show an autoradiogram of a SDS-polyacrylamide gel electrophoresis (PAGE) of each of the purified receptors used in the fusion experiments. As can be seen, the purification procedures yield receptor preparations of apparent homogeneity. The receptor peptides can be visualized by ¹²⁵I-iodination of the protein or by specific labelling of the preparations with the β -adrenergic photoaffinity probe ¹²⁵I-*p*-azidobenzylcarazolol, indicating that the isolated peptides do contain the ligand binding site of the receptor. It is clear that in all cases, insertion into phospholipid vesicles and fusion of a pure receptor preparation with the acceptor cell conveys a marked sensitivity of the adenylate cyclase of the hybrid membranes to isoprenaline. This stimulation is completely abolished by the β -antagonist propranolol and is not observed when cells are fused with themselves or when mixtures of phospholipid vesicles alone (that is, lacking β -receptors) are fused to the acceptor cells. The stimulation by isoprenaline in the fused hybrids is comparable with, or exceeds, that by the endogenous PGE₁ receptors, although the absolute extent of the adenylate

cyclase activities varies in different experiments. Similar degrees of isoprenaline stimulation of adenylate cyclase activity, per amount of reconstituted receptor added to the system, are obtained when the procedures for insertion of receptor into phospholipid vesicles are performed with a light vesicle fraction of frog erythrocyte lipids (see Fig. 3) instead of soybean phosphatidylcholine. These results demonstrate that the various polypeptides isolated contain the structures responsible for the activating function of the receptor (that is, presumably the ability to interact with the nucleotide binding protein of the adenylate cyclase system) in addition to the ligand binding site.

The extent of the stimulation by isoprenaline appears directly dependent on the amount of receptor inserted into phospholipid vesicles which is used in the fusion experiments. Essentially a linear response is observed over the range of receptor concentrations examined (150–1,500 fmol, data not shown). Similar responses are obtained with receptors isolated from frog erythrocytes, hamster lungs and guinea pig lungs, suggesting that the functional coupling of receptors from these different preparations to the nucleotide regulatory protein and adenylate cyclase of *X. laevis* erythrocytes is essentially identical. Moreover, the overall responses to the pure receptors are comparable with

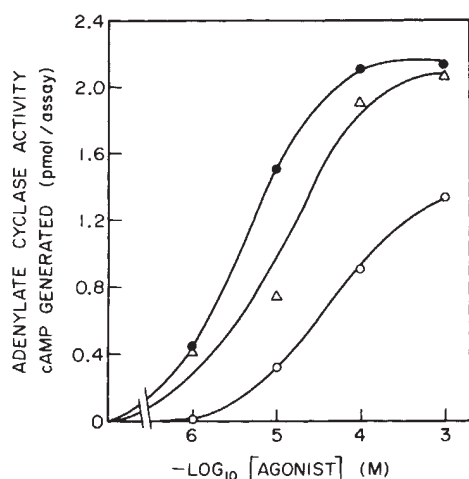


Fig. 3 Dose-response curves for agonist stimulation of adenylate cyclase in hybrid cell membranes. ●, (—) Isoprenaline; △, (—) adrenaline; ○, (—) noradrenaline.

Methods: Membranes were prepared from hybrids resulting from the fusion of 750 fmol of pure β -adrenergic receptor inserted into phospholipid vesicles (isolated from frog erythrocytes) and 2×10^7 *X. laevis* erythrocytes. Pure β -adrenergic receptor was inserted into a mixture of a light vesicle fraction of frog erythrocyte lipids (0.05 mM in phosphate) and DMPC (0.3 mM) as described in Table 1 legend (except that the addition of polyethylene glycol was omitted). The light vesicle fraction of frog lipids was prepared as reported earlier^{14,33}, essentially by centrifuging the 40,000g supernatant derived from a lysate of frog erythrocytes at 158,000g for 1 h. The resultant pellet contains ~ 0.05 μ mol lipid per mg protein (as determined by the method of Ames and Dubin²⁷ using DMPC as a standard) but negligible quantities of β -adrenergic receptors, nucleotide regulatory protein or adenylate cyclase. Fusion of the reconstituted receptor to *X. laevis* erythrocytes was performed as described in Fig. 2 legend except that 15 μ l of a phospholipid mixture of lecithin (200 μ g) and lysolecithin (10 μ g) were added before fusion with polyethylene glycol. Pure frog β -adrenergic receptor was prepared as described in Fig. 1 legend. The data shown represent duplicate determinations and are representative of two experiments.

those reported earlier for preparations partially purified by affinity chromatography¹⁴. This indicates that the capability for coupling to adenylate cyclase is not altered during the receptor purification procedures.

Specificity of responsiveness

In the present studies (Fig. 2), reconstitution of β -adrenergic responsiveness was accomplished using receptor preparations derived from tissues containing the β_2 subtype of receptors. Thus, it was of interest to assess whether the specificity of the catecholamine responsiveness conferred to the acceptor cells by the pure receptors would be conserved. Figure 3 presents catecholamine dose-response curves for the stimulation of a hybrid adenylate cyclase created by fusing pure frog erythrocyte β -adrenergic receptor to *X. laevis* erythrocytes. As can be seen from the data, the potency of these various agonists is isoprenaline > adrenaline > noradrenaline. The order of potency and the affinity of these agonists are essentially the same as those for β -adrenergic stimulation of frog erythrocyte adenylate cyclase or for competition of antagonist binding to frog erythrocyte membranes¹⁰. These results suggest that the pure receptor polypeptide is the determinant which confers not only the hormone stimulation of adenylate cyclase but also the specificity of the response.

Discussion

This report describes the first successful functional reconstitution of a pure receptor protein with an acceptor adenylate cyclase system. These results demonstrate that in the case of the β -adrenergic receptor, a single type of polypeptide chain (MW

58,000–64,000) contains both the binding site for catecholamines and the necessary sites for interaction with the nucleotide regulatory protein (N or G/F) and thus for activation of the effector adenylate cyclase. The results further underscore the fact that the affinity chromatography and HPLC procedures developed for purification of β -adrenergic receptors lead to the isolation of 'biologically active' receptor proteins. It is interesting that of the few receptors whose structures have been studied in detail, only the adenylate cyclase coupled β -adrenergic receptor appears to be purified as a single type of polypeptide. For example, the nicotinic cholinergic^{17,18}, insulin^{19,20} and IgE^{21,22} receptors all appear to be isolated with multiple associated subunits. However, in each of these cases a single subunit appears to carry the ligand binding site^{17–19,21}. Therefore, the apparent simplicity of the structure of the β -adrenergic receptors, compared with these other receptors, may in part stem from the fact that a distinct component—the nucleotide regulatory protein—mediates the signal transfer from the receptor to the effector adenylate cyclase.

Recently, various groups have used reconstitution and fusion methodology to study the functions of unpurified β -adrenergic receptor preparations. Eimerl *et al.* reported the fusion of crude soluble preparations containing β -receptors with cells containing adenylate cyclase but lacking receptors¹⁵. They established that receptors from one cell could confer catecholamine responsiveness to another cell. This had also been shown using cell-cell fusion^{23,24}. Subsequently, Pederson and Ross reported the coupling of unpurified β -receptors with the pure nucleotide regulatory protein via co-reconstitution of these proteins into lipid vesicles²⁵. These studies demonstrated the distinctiveness of the different components of the adenylate cyclase system and also provided the initial demonstration that reconstitution and fusion techniques represented a feasible approach to structure-function studies of the different adenylate cyclase components. More recently, we reported the first functional reconstitution of affinity chromatography purified β -adrenergic receptors with the *X. laevis* erythrocyte adenylate cyclase¹⁴, which opened the way to addressing the issue of reconstitution of pure receptor proteins. As noted above, the results described here prove the bifunctionality of the isolated β -receptor proteins, that is, these macromolecules contain both a ligand binding site and a site responsible for transmitting a biological signal (presumably via interactions with the nucleotide regulatory protein). However, other important implications of these studies relate to the further experimental questions which can now be approached. This reconstitution approach can be used to study: (1) the interaction of receptor and N, (2) the structural domains of the receptor involved in the binding and activating function of the receptor and (3) the structural basis of alterations of the activating function of receptors modified by physiological perturbations such as desensitization (which may lead to covalent modification, that is, phosphorylation of the receptor²⁶).

The ability to assay the binding function of adenylate cyclase coupled receptors over the past decade has led to considerable information about how such receptor molecules recognize and bind ligands. The development of these new reconstitution approaches should open the way to equally exciting insights into how hormone binding to receptors is translated into a biological response.

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LETTERS TO NATURE

Radio discovery of a young supernova

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It has become clear in the past few years that young supernovae can become rather powerful radio sources at an early stage of their evolution. Three young supernovae have been detected so far: SN1970g in M101 (ref. 1), SN1979c in M100 (ref. 2) and SN1980k in NGC6946 (refs 3, 4). The data on these objects and on older supernova remnants suggest that the radio emission turns on between 1 week and 1 yr after the supernova event, and that the supernova continues to be a radio emitter for at least a few years. About 50–100 yr later, when the supernova shell has swept up enough interstellar gas, the remnant may become visible again in the radio domain⁵. Previous searches for radio emission from young supernovae have been based on optically discovered supernovae. However, with the many detailed maps of the radio continuum emission from galaxies now available, it becomes feasible to find supernovae by comparing radio data alone. We report here the appearance of a radio source in the northern, inner spiral arm of NGC4258. Inspection of existing optical data (ref. 6, and W. L. W. Sargent and C. Kowal, personal communication) confirms that this source is a young supernova, making it the first radio detected supernova event.

We have three sets of observations on which the actual discovery⁷ of a radio supernova in NGC4258 is based: (1) a combination of data taken with the Westerbork Synthesis Radio Telescope (WSRT) in 1974–75 and data taken with the Very Large Array (VLA) in June 1979 at 1,415 and 1,480 MHz respectively⁸; (2) data taken with the VLA in January 1982 at 1,465 MHz as part of a systematic survey of a sample of Sbc galaxies; and (3) data taken with the WSRT between August and November 1982 at 1,416 MHz with the prime purpose of mapping the H I emission.

We have maps at comparable resolution (~13 arcs) from each of the three data sets. In addition, all observations have been calibrated on the scale of Baars *et al.*⁹ and can therefore be compared. Note that the 1982 VLA data suffer from missing short spacings and therefore miss extended radio emission with a scale size of 7 arc min or more. This is noticeable in the map as a varying zero level depression (see Fig. 1). In calculating the flux density of the radio source we have accounted for this. Additional, confirming observations have been made with the

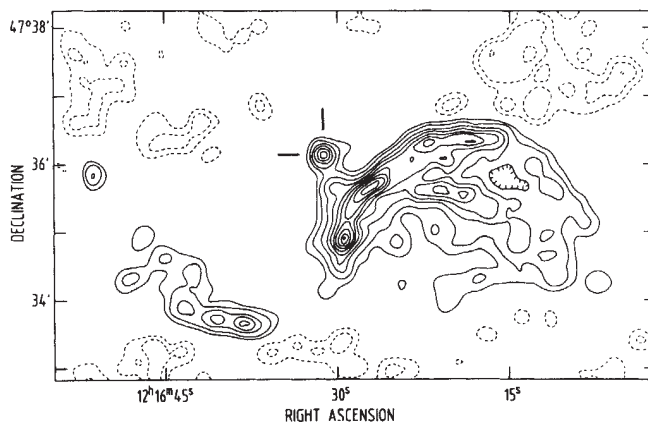


Fig. 1 Contour diagram of the January 1982 VLA map of NGC4258. The contours are -1, -0.5, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 mJy. The negative contours are dashed. The angular resolution is 14×14 arc s. The heavy lines indicate the position of the supernova.

VLA and the WSRT during May 1983. We will also briefly describe the results from these observations.

The January 1982 VLA map, displayed in Fig. 1, shows the anomalous radio continuum spiral arm structure¹⁰. A detailed comparison with the VLA-WSRT map of Van Albada and Van der Hulst⁸ (their Fig. 2) drew our attention to the 5.5 mJy, unresolved source at $\alpha(1950) = 12 \text{ h } 16 \text{ min } 31.23 \text{ s}$, $\delta(1950) = 47^\circ 36' 08.3''$, 1.3 arc min to the north and slightly to the east of the nucleus of NGC4258. Figure 3 shows an optical picture of NGC4258 with the position of the source indicated by an open circle. This source is not present in the VLA-WSRT map (flux density <0.25 mJy); also, a 6-cm WSRT observation made in May 1981 (A. G. De Bruyn, personal communication) does not show the source (flux density <1.3 mJy). To confirm this source we could turn to the WSRT H I line data obtained between August and November 1982. A map of the continuum emission, constructed from averaging channels free from line emission is shown in Fig. 2. The source is also present here but its flux density has apparently decreased to a level of 3 mJy. Its position agrees, within the observational errors, with the VLA position of this source. Table 1 gives the source parameters obtained from the observations described above.

From these radio observations of NGC4258 we can conclude that a source has appeared between May 1981 and January 1982. The independent VLA and WSRT detections are clear evidence that this source is real. A VLA observation of the source made in May 1983 (R. A. Sramek, personal communication) gave flux densities of 1.8 ± 0.4 mJy and 0.5 ± 0.1 mJy at 20 cm and 6 cm respectively. A May 1983 observation at 6 cm with the WSRT confirms this. These observations indicate that the source has an optically thin, non-thermal spectrum with a spectral index of $\alpha = 1.06 \pm 0.25$ ($S \propto \nu^\alpha$). Figure 4 shows all flux density measurements made to date at 20–21 cm as a

Table 1 Source parameter obtained from observations

Instrument	Frequency (MHz)	α (1950)	δ (1950)	Flux density (mJy)	Epoch
WSRT	1,415	—	—	<1	1972
VLA/WSRT	1,480/1,415	—	—	<0.25	June 1979/1974–75
VLA	1,465	12 h 16 min 31.23 s $\pm$ 0.15 s	47°36'08.3" $\pm$ 1.0"	5.5 $\pm$ 0.5	January 1982
WSRT	1,416	12 16 31.18 $\pm$ 0.2	47 36 08.2 $\pm$ 2.0	3.2 $\pm$ 0.5	August 1982
WSRT	1,416	12 16 31.18 $\pm$ 0.2	47 36 08.2 $\pm$ 2.0	2.8 $\pm$ 0.5	September 1982
WSRT	1,416	12 16 31.18 $\pm$ 0.2	47 36 08.2 $\pm$ 2.0	2.6 $\pm$ 0.5	October 1982

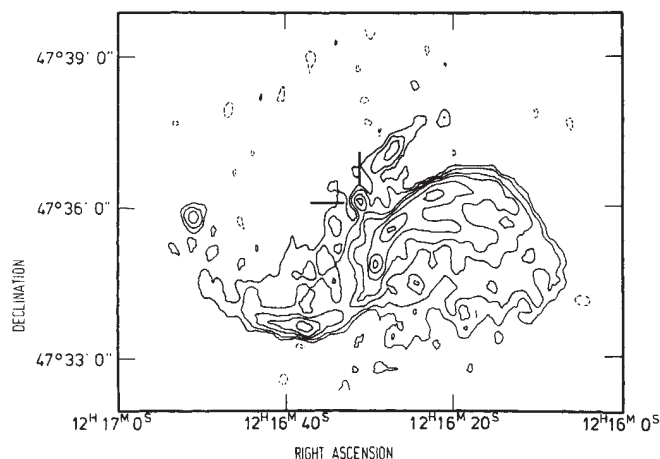


Fig. 2 Contour diagram of the August–October 1982 WSRT map of NGC4258. The contours are $-0.5, 0.5, 1, 2, 3, 5, 7.5$ and 10 mJy. The angular resolution is 14×18 arc s ($\alpha \times \delta$). The heavy lines indicate the position of the supernova.

function of time. It clearly suggests a steady decline in flux density at this wavelength. The e -folding time is 1.2 yr.

What is the nature of the source described above? Two possibilities come to mind immediately: it is an unrelated, highly variable background source, or it is an object located in NGC4258. We cannot exclude the first possibility, but think it is much more likely that the source is related to NGC4258, to be more specific, that it is a recent supernova. Our primary arguments for the hypothesis that this source is a young supernova are: (1) its location right next to a string of H II regions which outlines the inner, northern spiral arm; and (2) its sudden appearance as a radio source and the subsequent slow decline in flux density. The behaviour of the radio emission from this source is very similar to that of young radio supernovae⁴.

Optical evidence has also become available since the discovery of this source. NGC4258 is part of at least two ongoing optical supernova-search programmes. These are a programme by Wild⁶ who reported a 17th magnitude object within 2 arc s from the radio position on a November 3, 1981 exposure, and a programme by Sargent and Kowal (personal communication) who found a 16th magnitude object on a 1 August 1981 exposure. Sargent and Kowal did not see the supernova on either a 26 June 1981 exposure, or a 23 January 1982 exposure. Wild had a 5 July 1981 exposure with no detection and a 17 January 1982 exposure with only a very uncertain detection if any at all. The two certain optical detections suggest that the supernova reached its maximum in middle August 1981. From supernova lightcurves (see refs 11–14) one can infer that the maximum apparent brightness of this supernova was about 13th magnitude. The absolute magnitude of an average supernova is about -18 (for $H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$) (ref. 15). At the distance of NGC4258 one would hence expect an 11th magnitude supernova which is about 2 magnitudes brighter than our simple estimate of 13th magnitude. However, 1–2 magnitudes of absorption is possible in view of the supernova's location in the galaxy.

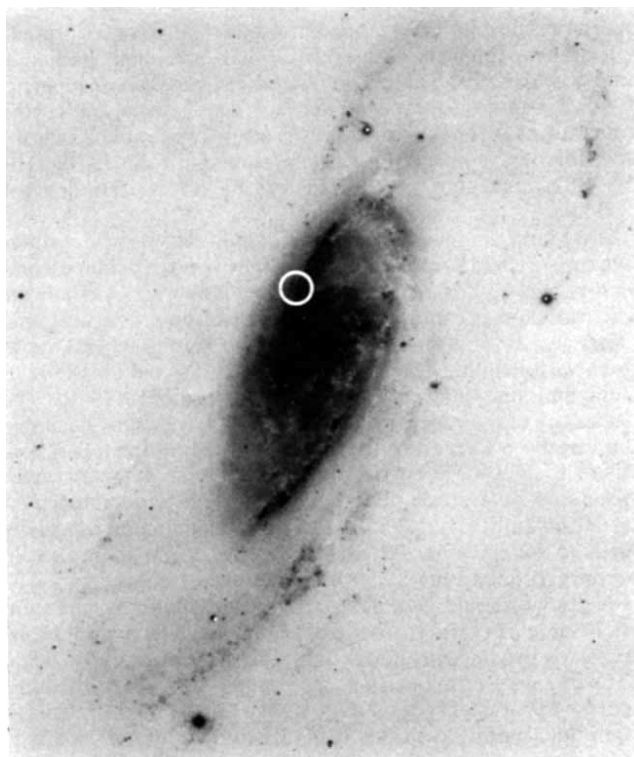


Fig. 3 Blue photograph of NGC4258 taken with the 5-m telescope at Mt Palomar Observatory by A. Sandage. A 30 arc s diameter circle is drawn around the supernova location in the inner spiral arm.

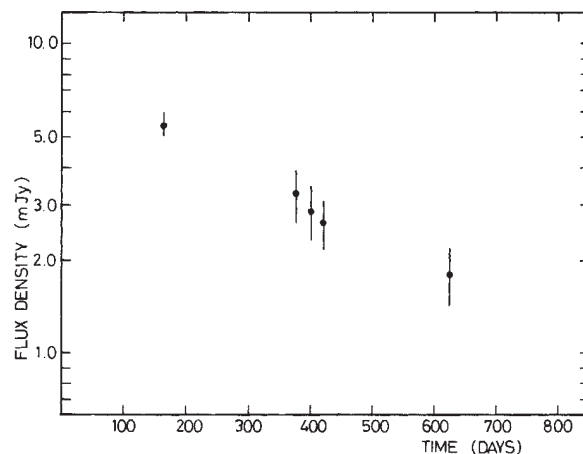


Fig. 4 Radio lightcurve of the supernova in NGC4258 at 20–21 cm. The origin of the time axis is 15 August 1981.

We, unfortunately, do not know whether this supernova was of Type I or Type II, but suspect it to be of Type II because of its location in a spiral arm and because all other radio-detected young supernovae are Type II. It is interesting to compare this

Table 2 Supernova characteristics at 1.4 GHz

Source	Flux density (mJy)	Distance (Mpc)	Radio power (Hz ⁻¹)	Ratio to Cas A
Cas A	2.2×10^6	0.0028	2×10^{18}	1
SN1970g	5.6	7.2	3×10^{19}	17
SN1979c	10.0	16	3×10^{20}	148
SN1980k	2.2	5	7×10^{18}	3
SN in NGC4258	5.5	6.6	3×10^{19}	14

supernova, despite the scanty knowledge we have at present, with other extragalactic supernovae that have been studied at radio wavelengths. Table 2 gives the characteristic properties of known radio supernovae and of the galactic supernova remnant Cas A for comparison. It is clear from Table 2 that the supernova in NGC4258 is very similar to SN1970g and SN1980k, but about an order of magnitude fainter than SN1979c.

As we have some idea of the radio 'light curve' of the supernova in NGC4258 we can make a slightly more detailed comparison. We do not have enough information about when the radio emission became detectable and can only conclude that it took less than 5 months for the radio source to emerge at 20-cm wavelength. This is similar to SN1970g and SN1980k at 20 cm, but quite different from the slower rise of SN1979c. The flux density of the supernova in NGC4258 was already declining significantly ~ 1 yr after the supernova explosion. This fast decline is quite different from the behaviour of the previous three radio supernovae. These did not begin to decrease their flux density until about 2–3 yr after optical maximum; especially SN1979c is very different: it has not yet begun to decline in February 1983, a time 3.8 yr after the explosion.

Finally we should comment on why this supernova was not first detected optically. The reason obviously is twofold: the supernova reached maximum light at a time of the year when NGC4258 was setting early in the evening and, secondly, most supernova search programmes do not go fainter than about 16th magnitude, unless, as in this case, attention is drawn to a particular position in a particular object. The present radio detection of a young supernova emphasizes the selection effects in supernova statistics.

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CCD images of the 6.1 ms pulsar field

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In a study of the radio pulsar¹ of period 6.13369 ms, the second shortest pulsar period known, we have obtained deep images Hof the field in different colour bands using the European Southern Observatory charge-coupled device camera at the 1.5 m Danish telescope at La Silla. At least three objects are visible inside the radio error box, and their astrometry and photometry are reported here.

The short-period end of the pulsar distribution is dominated by the Crab and Vela pulsars: they are typical young objects with a vigorous energy output, and, because of their unique evolutionary status, have served as a basis for much of the theoretical debate on pulsars' emission mechanisms (see, for example, ref. 2).

On the other hand, the recently discovered³ PSR1937+214, $P = 1.55781$ ms, appears as an extreme example of a different class of pulsars, which was already previously suspected⁴. Members of this class have also a very short period, but their period derivative $\dot{P}$ and energy output $\dot{E}$ are orders of magnitude lower than expected. This implies an extremely weak surface magnetic field, a very long spindown age, and presumably a very different evolutionary history^{5,6}. For PSR1937+214 an optical candidate ($m_R \approx 20$) with some indication of variability was announced by Djorgovsky⁷. The identification has been questioned^{8,9} because of the colours (consistent with a K giant reddened by 8 magnitudes) and of a small, but probably significant offset from the radio position. At any rate, high time-resolution photometry obtained at the Anglo-Australian Telescope through a 3.5 arc s diaphragm centred on Djorgovsky's star showed a 1% modulation of the signal with the radio period⁹. This is by and large consistent with a theoretical prediction of Pacini and Salvati¹⁰, which was normalized to the Crab and tested only with Vela. The indication is that the optical output depends mainly on the present values of P and $\dot{P}$ and does not keep memory of possibly different evolutionary paths.

Still more recently, during a search for pulsating objects inside COS-B γ -ray error boxes, the new fast pulsar PSR1953+29 was discovered, with $P = 6.13369$ ms¹. Pulse arrival time analysis indicates that the object is part of a binary system with $P_{\text{orb}} = (120 \pm 4)$ days. The published Very Large Array position is in error by one time second (R. Buccheri, personal communication), and the correct position within approximate 3σ limits is

$$\alpha(1950.0) = 19 \text{ h } 53 \text{ min } 26.7 \pm 0.24 \text{ s}$$

$$\delta(1950.0) = 29^\circ 00' \text{ min } 42.0 \pm 3.2 \text{ arc s}$$

The distance to the pulsar can only be estimated on the basis of the observed dispersion measure (1): with a free electron density of 0.03 cm^{-3} , $D = 3.5$ kpc is obtained, and the visual extinction A_v is expected to be several magnitudes. The only available information on $\dot{P}$ is an upper limit of $5.8 \times 10^{-16} \text{ ss}^{-1}$ (ref. 1). However, a very low surface magnetic field is already implied, while the binary nature provides additional evidence¹¹ that PSR1953+29 belongs to the same 'unorthodox' class as PSR1937+214. This very preliminary classification, and the expected magnitude deduced from the theory ($m_v > 17 + A_v$ at the nominal distance of 3.5 kpc) both point to the importance of a deep optical search in the field of the new pulsar, even if serious difficulties are presented by the poor accuracy of the

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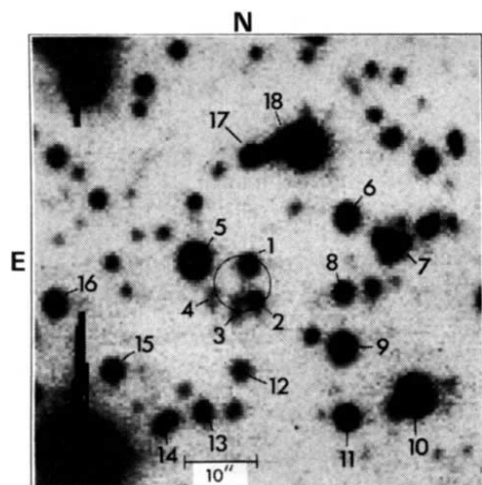


Fig. 1 Unfiltered CCD image: the radio error box is marked. The image was compared with pictures taken in Gunn Z and Johnson V colour bands. Seeing for the three bands was 1.6, 1.8 and 2.2 arc s respectively. Standard stars used were Ross 780 with Z magnitude 7.87 and, for V magnitude, Feige 67 with $M_U = 11.83$.

radio position and the high density of stars ($l = 65.84^\circ$, $b = +0.44^\circ$).

On a glass copy of the red Palomar Sky Survey three objects are visible near the radio position: two of these are star-like (marked with 1 and 5 in Fig. 1), while one (the superposition of 2 and 3 in Fig. 1) appears complex or extended. A print from the Palomar Schmidt Infrared Survey of the galactic plane shows all three objects at approximately the same relative intensities as on the red POSS copy; on the blue POSS plate, instead, only object no. 5 is visible. We have used the S3000 machine at the European Southern Observatory (ESO) Garching, to perform astrometry on the red POSS plate. Measurements of 14 stars from the South African Observatory catalogue resulted in random errors of 0.3 arc s in both coordinates, and the derived positions for several objects near the pulsar position are listed in Table 1.

To study their nature and their possible association with the radio pulsar, we obtained deep images using the ESO CCD

camera at the 1.5 m Danish telescope at La Silla (pixel size 0.47 arc s). The pictures were taken in three different colour bands—unfiltered light (Fig. 1), Gunn Z (900–1,000 nm) and Johnson V. The first two images have a better resolution than the POSS copies, and are estimated to be about two magnitudes deeper than the red copy. The V image was obtained at a large zenith distance and has a lower resolution than the others. The images have been reduced with the ESO IHAP software, and, in particular: flat field corrections were done with exposures of scattered light on the dome interior; no filtering or smoothing was applied; photometric calibration was achieved via observation of bright, defocused standard stars. The coordinate system of the CCD picture was established by means of the astrometric measurements referred to previously, with estimated zero-point errors <0.5 arc s. The resulting positions for numbers 2, 3 and 4 are also given in Table 1, together with V and Z magnitudes of all the numbered objects.

At least three of them fall inside the radio error box, and a fourth one lies on its border. Numbers 1 and 2 are star-like, while 3 and 4 appear extended or complex. On the basis of the present data alone none of the candidates appears conspicuous; a narrower radio position would be needed for a much deeper optical investigation. However, the time analysis of many hours of fast photometry is under way, and a better assessment of the problem should become possible.

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Searches for optical counterparts of the two millisecond pulsars

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Table 1 Coordinates and magnitudes for the objects in the field of PSR1953+29

ID no.	$\alpha(1950.0)$			$\delta(1950.0)$		m_V	m_Z
	h	min	s	arc min	arc s		
1	19	53	26.71	+29° 00'	43.9	20.1	17.8
2	19	53	26.65	+29 00	38.9	20.2*	18.1*
3	19	53	26.85	+29 00	37.7	—†	—†
4	19	53	27.10	+29 00	39.6	—†	19.7*
5	19	53	27.29	+29 00	44.3	17.5	16.8
6	19	53	25.68	+29 00	50.6	18.9	17.9
7	19	53	25.25	+29 00	46.6	19.3	16.9
8	19	53	25.72	+29 00	40.5	19.9	18.8*
9	19	53	25.74	+29 00	33.2	18.4	17.8
10	19	53	25.00	+29 00	26.4	16.7	16.1
11	19	53	25.69	+29 00	23.1	19.2	17.5
12	19	53	26.80	+29 00	29.3	20.3	19.0
13	19	53	27.24	+29 00	24.1	19.8	19.0
14	19	53	27.60	+29 00	22.2	20.0	18.7
15	19	53	28.14	+29 00	29.2	20.0	18.0
16	19	53	28.77	+29 00	38.5	20.6	16.7
17	19	53	26.64	+29 00	58.3	20.2*	17.5
18	19	53	26.14	+29 01	00.4	16.0*	15.7*

The astrometry of objects 1 and 5 to 18 is from a red POSS glass copy; objects 2, 3, 4 from the CCD unfiltered image.

* Magnitude poorly determined.

† Magnitude unmeasurable.

The first millisecond pulsar, 1937+214¹, has given rise to much observational and theoretical excitement (reviewed in ref. 2). A 20th magnitude red object was proposed earlier as a possible optical counterpart³, but we show here that this is not the case: this object is probably a giant star at a distance of 5–6 kpc. There is no evidence for an optical source brighter than the 23rd red magnitude at the improved optical position. We also present results of the search for the optical counterpart of the new millisecond pulsar 1953+290⁴. A grid of astrometric offset stars for this source is given, and possibility of optical detection discussed.

We have obtained a good quality spectrum of the candidate object³ (Fig. 1) on the night of 9 May 1983 UT, using CRYOCAM charge coupled device (CCD) spectrograph on the Kitt Peak 4-m telescope. Very heavy extinction is evident. We have clearly detected H α in absorption, but the sodium D lines are very weak, if present at all. This is suggestive of an early-type star. Assuming a flat ($F_\nu = \text{constant}$) spectrum, we obtain $A_V = 9.6$ m; assuming that the object is a K-giant⁵, we derive visual line-of-sight extinction $A_V = 8.2$ m. This is in good agreement with the estimate of Middleditch *et al.*⁶. It also corresponds to a distance of 5–6 kpc, curiously close to the distance to the pulsar, found by the radio methods⁷.

New deep CCD exposures were obtained on the night of 5 July 1983 UT, on the Lick 3-m telescope, in a wide red band-pass, with $\lambda_{\text{eff}} = 6,500 \text{ \AA}$ (r_{ccd}). Reevaluation of the position of

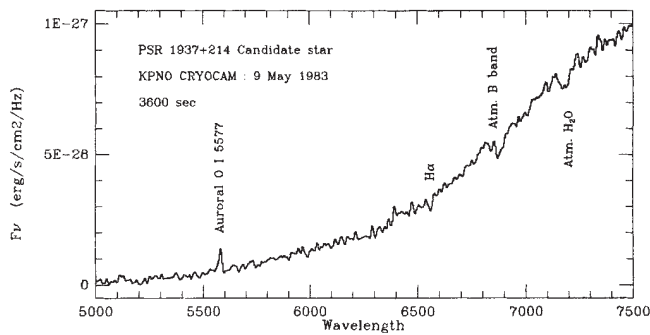


Fig. 1 Spectrum of the star originally thought to be the PSR1937+214. Atmospheric features and stellar H α absorption are marked. Note the absence of any significant sodium D lines ($\lambda = 5,893 \text{ \AA}$).

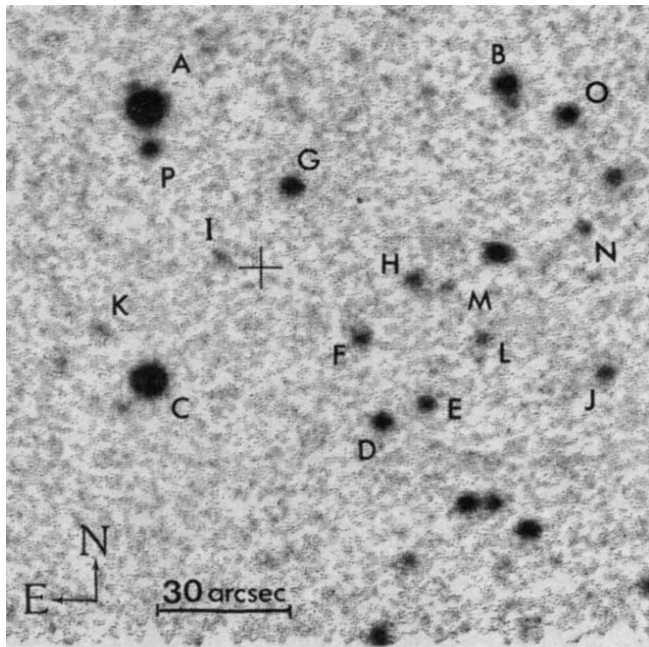


Fig. 2 PSR1953+290 field with astrometric offset stars labelled. The radio position of the pulsar is marked with the cross.

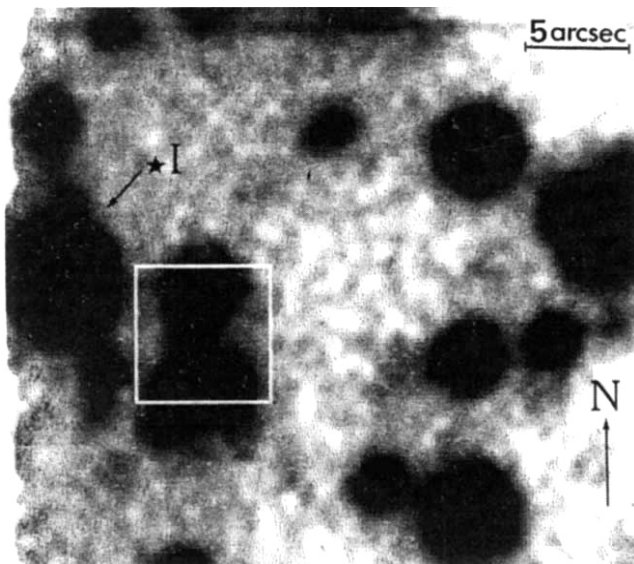


Fig. 3 A stack of the CCD frames of the PSR1953+290 field. White box is the radio position error box. Star I is listed in Table 1, and has magnitude $r_{\text{CD}} = 12.5$. The two stars bracketing the pulsar position have magnitude 14.4 (N) and 14.6 (S).

the red star shows that it is 0.4 arc s east and 2.9 arc s north of the radio position (with 1σ error of 0.5 arc s in each coordinate). The error in the original identification measurement seems to have been caused by an error in assumed CCD pixel scale, discovered since then (T. Lauer, personal communication). There is no optical source at the revised position with $r_{\text{CD}} \leq 23$. We have measured the red magnitude of this star to be 18.9 (July 1983), whereas it was measured to be 19.6 in October 1982, and greater than about 20 from the POSS E print (1950). This may constitute an evidence for variability.

Middleditch *et al.*⁶ have reported negative result of the search for optical and infrared pulses from (roughly) this location, whereas Manchester *et al.*⁸ reported tentative detection of the optical pulses, corresponding approximately to $r_{\text{CD}} = 24$. The pulsed-light estimates are still consistent both with our upper limit to apparent brightness, and the upper limit to pulse intensity from Middleditch *et al.*

The 6 ms pulsar, 1953+290, was discovered⁴ in the error box of a γ -source 2CG065+00. It is a member of a 120-day binary, at an estimated distance of 3.5 kpc. We have obtained multiple CCD exposures on the pulsar field on the nights of 16 and 17 May and 9 June 1983 UT, on the Lick 1-m telescope, and on 12, 13 and 14 June 1983 UT on the Lick 3-m telescope. In addition, two astrometric plates were taken on the night of 19 May 1983 UT on the Lick double astrograph by E. Harlan.

The astrograph plates were digitized on the Berkeley Photometric Data Systems microdensitometer, and the better one of the two was used to determine the equatorial positions in the field. First, some fifteen SAO stars were used to establish the equatorial coordinates on the plate, and $(\alpha, \delta)_{1950}$ positions were evaluated for the set of fainter stars surrounding the pulsar. They are listed in the Table 1, and identified on the Fig. 2. Several of those stars were then used to establish the position of the pulsar on the CCD frames. The astrometric 1σ errors are estimated to be 0.4 arc s in either coordinate; most of the error is contributed by the coordinate transformations. Figure 3 shows a section of a digital stack of several CCD exposures, with the radio position error box from Boriakoff *et al.*⁴. The two brighter objects are most likely foreground stars. There is a possible faint source in the middle of the error box, but proximity of the two much brighter stars just next to has foiled our attempts of image enhancement or photometry. Our estimate is that this object, if real, has a $r_{\text{CD}} \approx 22$ –23. Another possibility are the objects in the south-east corner of the error box. A model-dependent estimate of expected optical brightness can be made by using heuristic formula given by Kulkarni², which can be stated as:

$$m_{v,\text{est}} = 9.612 + 4.1084 * \log(P/\text{ms}) - 0.828 * \log(\dot{P}/10^{-19}) + 5 * \log(D/\text{kpc}) + A_v \quad (1)$$

If we use the measurements of Boriakoff *et al.* for the period P , their upper limit for the period derivative $\dot{P}$, estimate of the distance, and average extinction of 1.6 kpc^{-1} , we obtain $m_{v,\text{est}} \approx 18$ for an upper limit. So, even if $\dot{P}$ has a true value 100 times smaller than the measured upper limit, the pulsar may be detectable on our CCD frames. A similar (though slightly fainter) limit can be obtained by using the model by Pacini and Salvati⁹. One must also consider a possibility of additional light from its binary companion. Most theoretical scenarios offered so far^{10–12} predict that the other star is probably a white dwarf, unless the accretion phase had just finished, in which case it may be very luminous ($L \approx 300L_{\odot}$), and thus detectable. Possible nonthermal optical activity may also be expected if this binary is indeed the γ -source 2CG065+00.

Thus, there is a distinct possibility that the pulsar may be detected optically. Further pulse search and spectroscopic work is needed to check this proposition, but it will be difficult due to the presence of the much brighter foreground stars nearby.

We thank to Mr E. Harlan for taking the astrograph plates, Drs M. Lebofsky, G. Rieke, D. Cudaback and J. Middleditch for communicating results prior to publication and S. Kulkarni for stimulating discussions. We are greatly indebted to the staff

Table 1 Equatorial positions for the offset stars for PSR1953+290

Star	α (1950)	δ (1950)
A	19 h 53 min 28. 523 s	29° 01 arc min 18. 03 arc s
B	19 53 22. 602	29 01 23. 34
C	19 53 28. 445	29 00 17. 70
D	19 53 24. 602	29 00 08. 67
E	19 53 23. 891	29 00 12. 73
F	19 53 24. 969	29 00 26. 95
G	19 53 26. 109	29 01 00. 69
H	19 53 24. 102	29 00 39. 99
I	19 53 27. 250	29 00 44. 86
J	19 53 20. 945	29 00 19. 36
K	19 53 29. 227	29 00 28. 79
L	19 53 22. 969	29 00 27. 14
M	19 53 23. 547	29 00 38. 55
N	19 53 21. 313	29 00 51. 63
O	19 53 21. 594	29 01 16. 73
P	19 53 28. 453	29 01 09. 23

Estimated errors are 0.4 arc s.

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Evidence for disequilibrium of ortho and para hydrogen on Jupiter from Voyager IRIS measurements

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Specification of the ratio of molecular hydrogen in the ortho state (parallel proton spins) to that in the para state (antiparallel proton spins) has long been a problem encountered in the modelling of the atmospheres of the outer planets^{1–3}. At deeper atmospheric levels where the temperature exceeds 300 K, a 'normal' ortho-para ratio of 3:1 will exist; as a parcel is transported upwards to cooler levels, the ratio can either remain fixed or move toward the 'equilibrium' value at the local temperature, depending on the relative magnitudes of the equilibration and transport times, neither of which is well known. To make further progress in the investigation of these processes, it is important to determine the ortho-para ratios within the observable portions of the atmospheres of the giant planets. Here we present preliminary results of an analysis of the H₂ ortho-para ratio in the atmosphere of Jupiter using Voyager infrared spectrometer (IRIS) data.

Fig. 1 Thermodynamic properties as functions of temperature for a mixture of 90% H₂ and 10% He. Upper panel: temperature increment ΔT resulting from equilibration of normal H₂ at the indicated temperature. Lower panel: nondimensional specific heats for fixed para hydrogen fraction f_p compared with that for equilibrium H₂. The broken line is the case for which f_p is fixed at the equilibrium value for the local temperature.

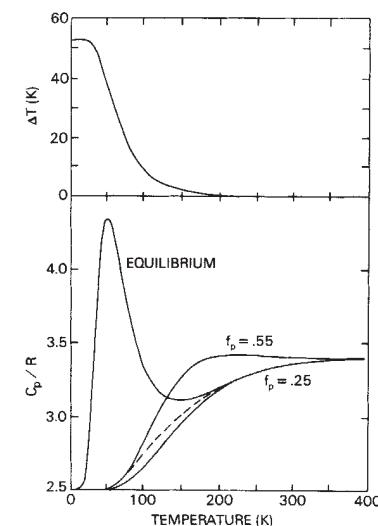
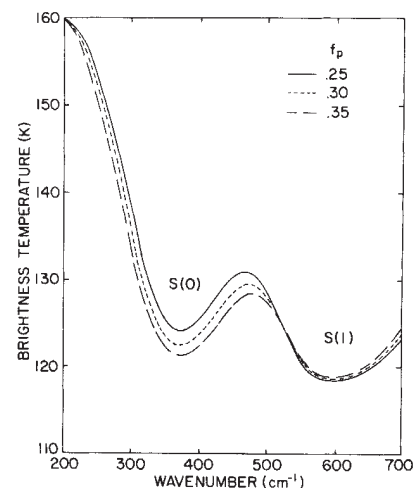


Fig. 2 Theoretical jovian thermal emission spectra calculated for the indicated height-independent values of f_p .



To illustrate the importance of understanding the thermodynamics of a predominantly H₂ atmosphere, specific heats which result for fixed ortho-para ratios are compared with the equilibrium value in Fig. 1. Also shown are the increments in atmospheric temperature which will result if parcels of normal H₂ are allowed to equilibrate at the indicated temperatures. These increments are of the same magnitudes as the horizontal temperature differences observed on Jupiter and Saturn. The equilibration time for pure hydrogen is known to be quite long ($\sim 10^8$ s), but can be greatly shortened through the action of catalysts. Recently Massie and Hunt⁴ have constructed one-dimensional models in which equilibration occurs through catalytic processes on the surface of atmospheric cloud particles, and Gierasch⁵ has demonstrated that it is possible to generate thermal contrasts which can yield wind speeds comparable to those observed on both Jupiter and Saturn.

The Voyager IRIS instruments obtained measurements of the thermal emission of Jupiter with an apodized resolution of 4.3 cm⁻¹ over a broad spectral range^{6–8}. The spectral region of interest for this study is 300 to 700 cm⁻¹ which is dominated by pressure induced H₂ lines. Since the S(0) line results from transitions between para energy levels while the S(1) is formed by transitions between ortho levels, the ratio of the line strengths is proportional to the ortho-para ratio. In general, the atmospheric thermal emission spectrum depends on both the fraction of H₂ in the para state f_p and the atmospheric thermal structure. However, as illustrated in Fig. 2, there is a crossover point between the two lines near 520 cm⁻¹ where the opacity is essentially independent of f_p , and in addition there is little sensitivity to atmospheric opacity near 600 cm⁻¹ because emission originates near the tropopause where the lapse rate is low. By combining measurements from the regions of low f_p

Fig. 3 Measured jovian thermal emission spectrum compared with theoretical spectra calculated for equilibrium f_p and for a height independent value of 0.3.

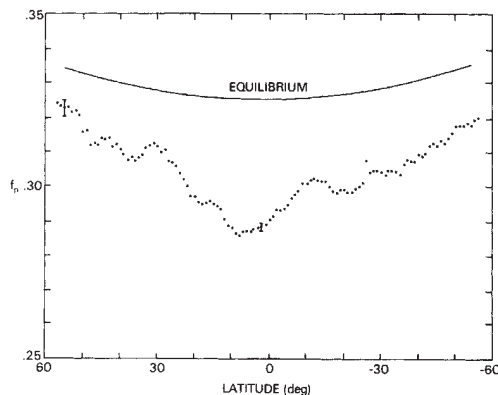
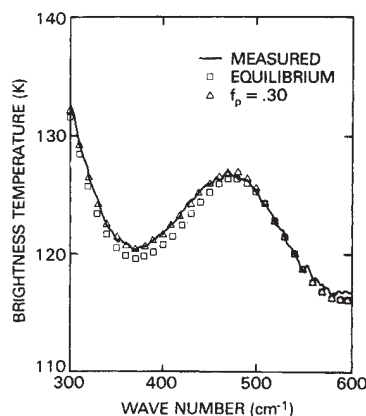


Fig. 4 Inferred para hydrogen fraction f_p as a function of latitude. The values shown represent running averages of the data over intervals 5° wide calculated at increments of 1° . If f_p assumed the equilibrium value at each atmospheric level the results would lie along the solid curve.

sensitivity with those from other regions of similar atmospheric optical depth which depend strongly on f_p , it is possible to estimate both temperature and the ortho-para ratio.

Previous efforts at modelling this portion of the spectrum have assumed equilibrium H_2 and have not succeeded in fitting the measurements to the instrumental noise level⁹. Reexamination of the problem indicates that relaxation of the constraint of equilibrium f_p permits better fits to be obtained as indicated in Fig. 3. In this example, the Voyager 1 ingress radio occultation profile was used, and the required H_2 absorption coefficients were based on the work of Birnbaum and Cohen¹⁰. To account for He- H_2 collisional contributions to the opacity, a He mole fraction of 0.10 was utilized⁹. A height independent $f_p = 0.30$ is found to give a better fit than equilibrium H_2 .

To investigate further the possible ortho-para disequilibrium, an algorithm was formulated for the rapid estimation of f_p from individual spectra. The f_p -insensitive regions near 520 and 600 cm^{-1} were used to estimate a temperature and lapse rate for the upper troposphere which were then used to estimate f_p by comparison of theoretical and measured brightness temperatures near 330 cm^{-1} where the nominal optical depth is comparable to that at 520 cm^{-1} . This procedure results in an inferred equivalent constant f_p for an atmospheric layer approximately one scale height thick centred near the 250 mbar level.

The technique was applied to approximately 600 spectra from a Voyager 1 sequence of data which mapped Jupiter over 360° of longitude between $\pm 60^\circ$ latitude. The results are displayed in Fig. 4 where the individual data points have been subjected to a running average 5° wide in latitude. The value of f_p which would be obtained for equilibrium H_2 is included in the figure for comparison; its value varies with latitude because the emission angle of the observations varies, and slightly different portions of the equilibrium f_p profile are sampled. The results indicate a strong large scale latitude dependence with superposed smaller scale structure. The minimum lies slightly north

of the equator and has a value approximately halfway between equilibrium and normal.

Errors resulting from the propagation of instrument noise are relatively small for the running means, as indicated in Fig. 4. Absorption coefficient errors could produce a systematic error in the inferred f_p , but to account for the apparent departure from equilibrium at low latitudes, a differential error in the total absorption coefficient of $\sim 15\%$ would be required which seems unlikely. In addition, the latitude dependence could not be explained by such an error. The most difficult source of uncertainty to evaluate is that due to clouds or hazes which can contribute to the atmospheric opacity^{11,12}. The inferred values of f_p have been compared with measurements within spectral 'windows' where the gaseous opacity is relatively low at 225 cm^{-1} and 2,000 cm^{-1} and with measurements from the reflected solar radiometer of the IRIS instrument. None of these potential indicators of cloud behaviour correlates consistently with the inferred f_p . We conclude that the most plausible interpretation of the results presented here is that the ortho-para ratio departs from equilibrium in the upper troposphere of Jupiter and displays a strong variation on a planetary scale.

The observation of f_p values which are intermediate between normal and equilibrium implies that the equilibration and dynamic time scales are comparable in magnitude. In these conditions, the large energies associated with equilibration will significantly enhance the efficiency of atmospheric energy transport processes. The observed disequilibrium may also possess diagnostic value; since it is associated with vertical motion, high resolution mapping of f_p may facilitate mapping of the atmospheric flow field. Finally, the results indicate a need for a precise knowledge of hydrogen equilibration times, and further work is required in this area, especially with regard to surface processes on NH_3 ice cloud particles.

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Plate rupture by hydraulic fracture resulting in overthrusting

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Lithospheric plates, bending into subduction zones, may become ruptured by hydraulic fracturing initiated by the injection of mobilized sediments. Thick, poorly indurated, water-laden sediments of the proximal continental rise are the most obvious potential source for the large volume of material required for this process. Rupture of the descending plate may result in the subducted oceanic part of the lithosphere becoming detached and sinking independently, while the more buoyant continental part rises by isostatic rebound. We show here that

applying this model to eastern Indonesia provides an explanation for the Banda Arc geometry, the apparent absence of a normal forearc and for the position of the thrust sheets relative to the Benioff zone¹. We also report that it can account for the short phase of deformation of the Australian continental margin preceding its uplift of 3 km (ref. 1).

The problem of how the oceanic part of the descending slab, beneath the collision zone may detach and sink resolves itself into two distinct aspects. These are: (1) in order that detachment may take place it is necessary to explain how an extremely extensive fracture or plane of weakness, of the correct orientation, may develop in the descending lithosphere, and (2) although such a plane of weakness is necessary, it is not of itself sufficient to allow detachment to take place; it is therefore also important to identify a mechanism which will actuate detachment along such a plane of weakness.

Mathematical modelling of the bending lithosphere, based on elastic theory has attempted to explain the observed bathymetric profiles associated with such features as oceanic islands, island chains and arcs and ocean trenches². The models are essentially two dimensional and assume a significant portion of the lithosphere can be treated as a homogeneous and isotropic, linear elastic layer, floating on a viscous mantle. An expression has been derived which defines the universal flexural profile, which was fitted to the bathymetric profile across the Mariana Trench. By choosing suitable and reasonable elastic moduli [$E = 7 \times 10^5$ bar and $m = 4.01$] a thickness of 28 km for the elastic lithosphere can be obtained, which is in good agreement with the estimated thickness of the elastic lithosphere derived from deflections resulting from loading of the lithosphere by an oceanic island³.

Turcotte and Schubert² noted that the largest bending stress obtained from this analysis occurred 20 km seaward of the trench axis where it attained a magnitude of 9.0 kbar. It can be shown that if the elastic lithosphere has a thickness of 28 km and using the values for E and m already quoted, then throughout the whole of the Mariana Island Arc, the outer fibres of the elastic lithosphere would tend to experience a tensile stress of almost 3.0 kbar. These bending stresses would be superimposed on the existing rock stresses. For example, the lithosphere may be in a state of general horizontal compression. It has been argued that in such a general compression, the average differential stress is not likely to exceed 1.0 kbar (ref. 4). Hence, the rocks of the outer fibres would experience a significant lateral reduction in stress that has the potential of attaining a total tensile stress of -2.0 kbar.

From rock mechanics data⁴ it is clear that near-surface rocks could not sustain a tensile stress of -2.0 kbar without failure. Indeed, the upper levels of the lithosphere in the vicinity of the island arc would develop normal faults, before the total lateral stresses actually became tensile.

These estimates of stress are based on the assumption that, initially, the neutral surface is in the middle of the so-called elastic portion of the plate (at a depth of 14 km). There would be perhaps 70 or more kilometres of the lithosphere that would exercise an influence (possibly even an elastic influence) on the deformation of the lithosphere, so that the real neutral surface is likely to be deeper than 14 km: and hence the tensile fibre stresses would be correspondingly higher. Just as the outer fibres of the elastic lithosphere would undergo extension, the inner fibres would experience compression and tend to develop a compressive differential stress that could potentially reach 3.0 kbar. If, in addition, the lithosphere was experiencing a general horizontal compression, these bending stresses would augment the general level of the differential stress.

From the geometry of the bathymetric profile and an assumed rate of plate convergence (10 cm yr^{-1}) one may infer that the average strain-rate in the inner fibres (at a depth of 28 km) must be of the order of 10^{-14} s^{-1} . From rock mechanics data⁴ one may infer that, at temperatures of perhaps 700°C and the stated rate of strain, the rocks would yield in a ductile manner, long before the differential stress attained its maximum potential value.

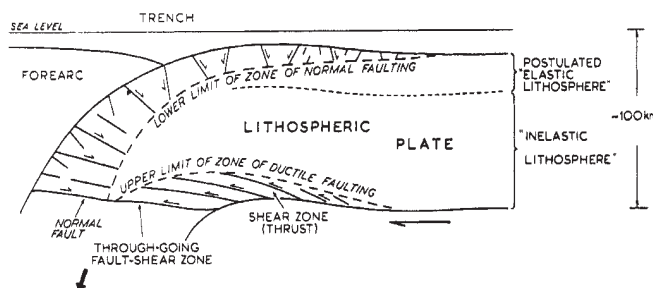


Fig. 1 Diagrammatic representation of development of normal faults in the upper zones, and thrust shears in the lower zones of an oceanic lithospheric plate, which may lead to through-cutting planes of weakness.

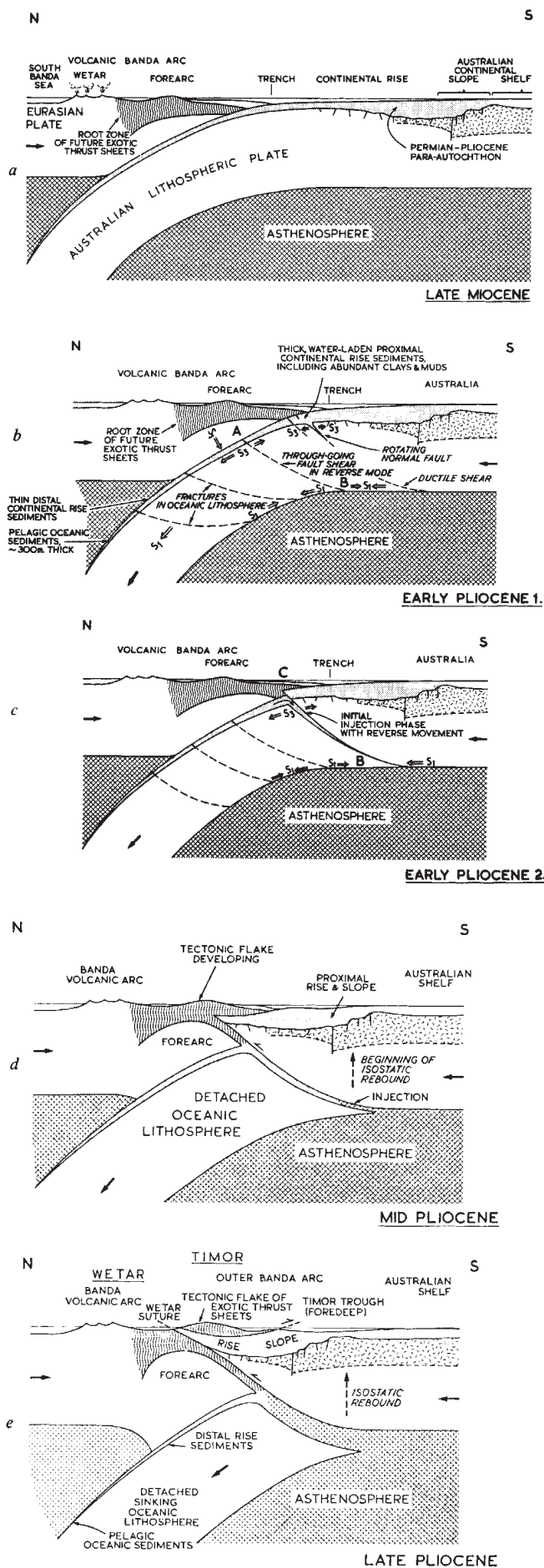
Where deep crustal rocks have been uplifted and exposed by erosion, it is commonly noted that significant deformation has resulted from the generation of ductile shear zones. Even though this type of failure is clearly non-brittle, nevertheless (from the acute angle between conjugate shears coupled with the sense of displacement) it is often possible to interpret such shears in terms of principal stress orientation in exactly the way in which one can interpret brittle shears^{5,6}. Consequently, one may infer that the ductile deformation of the lower levels of the former elastic layer under consideration would probably result, in part at least, in the development of ductile shear zones. The geometry of the collision line of the Australian continental plate with the southward curving portion of the Banda Arc would ensure that such shear zones, in this locality, would have the orientation of thrusts.

The failure at the outer and inner fibres, by brittle normal faulting and ductile shear, respectively, causes thinning of the elastic portion of the lithosphere. The curvature of the lithosphere tends to increase progressively towards the collision line. Consequently, there will be a corresponding tendency for the fibre stresses to become higher as this line is approached. Such a tendency results in the normal faults becoming extended to deeper levels and the ductile shear zones to extend into higher levels of the former elastic portion of the lithospheric plate. That is, the elastic portion of the plate becomes effectively thinner as the trench is approached (Fig. 1).

When the elastic portion of the lithospheric plate becomes very thin, thrusts may extend and join with normal fault planes. As the plate descends into the trench, these normal faults would have a relatively low dip. The coalescence of these structures would result in a major plane of weakness. Continued movement on the thrust could cause the normal faults portion of the combined structure to exhibit a reversal of movement, so that the whole plane of weakness could become a thrust.

The existence of only one such plane is indicated in Fig. 1. However, it would be expected that, as the oceanic portion of the plate was flexed near the collision line, many such fractures would have developed in the down-going slab. It is suggested that separation and detachment do not usually occur at these fractures in the oceanic plate; the upper part of the descending oceanic plate has the potential to sink faster than the lower portions of the descending plate. The slower rate of descent of the lower portion comes about because the slab will have become hotter and relatively less dense and also it will have become weaker and so begins to deform in a ductile manner at its leading edge.

The postulated major fractures will develop at depths of about 40–50 km (Fig. 1) that is at a depth where the vertical total stress will be 12–17 kbar. The descending slab cannot detach and drop away of its own accord, because the rocks above the fracture could not withstand such a large, unsupported, vertical load. The rock mass above would collapse and keep the fracture, or weakness plane, closed by following the main slab on its downward journey.



For detachment to take place, the fracture and plane of weakness must be forced apart and entered by a ductile material, which will be at high pressure and thus able to support the gravitational load of the lithosphere above the plane of detachment. The mechanism which can accomplish this is termed hydraulic fracture⁴. In its simplest form the hydraulic fracture criterion is given by $P > S_3 + T$, where P is the pressure of the injecting fluid, S_3 is the least principal stress and T is the tensile strength of the material (lithosphere). Fractures which form by this mechanism develop at right angles to the axis of least principal stress.

It may be inferred from the previous arguments that in the straight portions of the descending slab, the maximum compression (S_1) will act down the length of the slab. This will inhibit hydraulic fracture and/or opening of fractures in this portion of the slab. However, where the lithospheric plate is undergoing flexure, S_3 will be large and aligned approximately parallel to the boundary of the plate, as indicated at point A in Fig. 2b. Hence, it is here, where the plate is flexuring, that hydraulic fracture is most likely to take place. Moreover, the lithosphere is already cut by large fractures (so that the tensile strength T is reduced to zero). Although these preexisting fractures may not be oriented exactly perpendicular to the least principal stress, it is likely that they will be opened by the 'fluids' rather than completely new structures be generated by the hydraulic fracture mechanism.

The asthenosphere will eventually act as the fluid which will occupy the widening fracture. However, as may be inferred from Fig. 2b, initially the asthenosphere is many tens of kilometres distant from the likely site of hydraulic fracture. (The high pressures which exist in the inner portion of the flexuring lithosphere, near point B in Fig. 2b, would prevent hydraulic fracture where, otherwise, the asthenosphere would have ready access.) Instead, for a suitable and locally situated fluid material we must turn to the large volume of sediments of the subducted continental margin. These relatively poorly indurated, water-laden, continental-rise sediments subjected to a total pressure of 10–30 kbar, with a commensurate high interstitial water pressure, would be able to sustain only a very small differential stress, and so would readily break down and flow as a fluid. Because of their low relative density these sediments would tend to rise in a diapiric manner. However, their upward motion can initially be blocked by high horizontal stresses, near point C in Fig. 2c, so that these weak sediments become instrumental in initiating detachment along the existing fault or weakness plane. Injection of these sediments could cause fractures to propagate through the lithosphere even if a suitable through-going plane of weakness did not already exist. It is possible that the weak sediments

Fig. 2 Application of model to eastern Indonesia (true scale). *a*, Late Miocene reconstruction⁷ of Banda Arc-Trench system before the Australian continental margin^{8,9} collided with the trench. *b*, Early Pliocene collision of continental margin with the trench^{7,10,11}. Reconstruction illustrates the development of fractures in brittle oceanic crust propagating downward through the lithosphere into ductile shear zones. Note the hydraulic fracture by injection of mobilized continental rise sediments and the role of reversed movement on rotated normal faults. *c*, Later in the early Pliocene¹¹ the upper, more buoyant part of subducting lithosphere begins to rupture aided by hydraulic fracture and reversed fault movement, so that the converging, Australian lithosphere begins to drive a wedge into the Banda forearc basement. *d*, Mid-Pliocene deformation of the Australian continental margin deposits and emplacement of exotic thrust sheets from the north^{7,11,12}. Oceanic lithospheric slab begins to sink independently, with the inflow of asthenosphere while the still converging Australian continent¹⁰ rides over the Benioff Zone and part of the Banda forearc region. The leading edge of the forearc region begins to detach as a tectonic flake¹³. *e*, Late Pliocene reconstruction, showing the Australian continental margin with thrust sheets emplaced, starting to rise by isostatic recovery^{1,14,15}. This uplift led to erosion of the rising 'outer' Banda Island Arc and deposition of post-orogenic molasse¹¹ on the northern side of the developing Timor Trough foredeep.

may begin to infill the normal fault planes, as they rotate, at depths of as little as 10–20 km below the surface, and that this mechanism could contribute to the development of through-going fractures.

The subsequent widening and infilling of the detachment zone by material from the asthenosphere are depicted in Fig. 2c–e, where we illustrate the application of the proposed lithospheric rupture mechanism to the Banda Arc by a series of true scale diagrams.

Obvious implications arise from this mechanism. For example, mixing and inclusion of fragments of lithosphere and even asthenosphere into the mobilized sediments is to be expected: and these may eventually be brought to the surface by diapiric action. Also, free water may locally pass from the mobilized sediments into the initially dry deep lithospheric rocks.

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Evidence for photochemical generation of superoxide ion in humic waters

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On irradiation with visible or near UV light the coloured substances in natural humic waters can undergo several reactions. They may be bleached and partially degraded^{1–3}, perhaps by a cyclic mechanism involving oxidation and reduction of iron⁴, or they may generate a variety of free radicals⁵ or serve as sensitizers for the generation of singlet oxygen^{6–8}. They may also bring about the generation of hydrogen peroxide^{9–11}. It has been suggested^{10,11} that the actual photochemical process may be the generation of superoxide ion, and that hydrogen peroxide is produced by the dismutation of this. We present here more direct evidence for the photochemical generation of superoxide ion, $O_2^{\cdot-}$, in natural waters.

The superoxide ion is the first product of the reduction of the dioxygen molecule. It is unstable in aqueous solution, undergoing a rapid disproportionation to hydrogen peroxide and dioxygen. In many types of cells this reaction is hastened by specific enzymes, superoxide dismutases (SOD)¹². The superoxide ion can act either as a reductant or as an oxidant¹³. As an oxidant, it can oxidize hydroxylamine to nitrite; because nitrite can be measured spectrophotometrically at low concentrations this reaction provides a sensitive and convenient means of measuring superoxide¹⁴.

We have observed photochemical formation of nitrite from hydroxylamine in samples of humic water from two sources, Wylde Lake, Ontario and Lac Cloche, Quebec, containing 30–100 mg l⁻¹ and about 10 mg l⁻¹ of dissolved organic carbon respectively⁸. Most experiments were performed using Wylde Lake water as it was more active. A typical result is shown in Fig. 1. In the conditions of the experiment the rate of nitrite

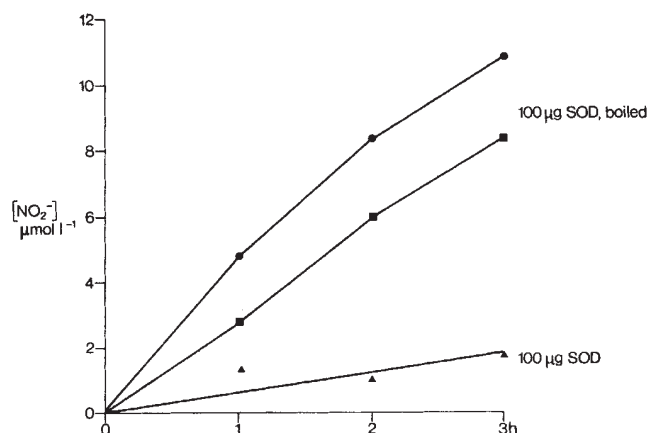


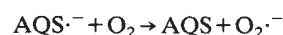
Fig. 1 Photochemical oxidation of hydroxylamine to nitrite in humic water and its inhibition by superoxide dismutase (SOD). [Type I, from bovine blood, 2,750 units mg⁻¹ (Sigma).] The reaction mixture contained 50% Wylde Lake water, 10⁻³ M hydroxyl ammonium chloride and 0.02 M potassium phosphate pH 7.4 (●); with the addition of 5 μg ml⁻¹ SOD (▲) or 5 μg ml⁻¹ SOD inactivated by boiling (○). Samples were placed in a 10-ml quartz cuvette supported in a holder cooled with tap water to about 9 °C. Light from a 200-W Hg–Xe bulb was focused through a Pyrex glass filter onto the face of the cuvette. The light intensity at the face was about 250 mW cm⁻². The reaction mixture was stirred with a Teflon-coated magnetic stirring bar driven by a magnetic stirrer mounted against the face of the cuvette opposite to the light source. Samples (1 ml) were removed at intervals for nitrite determination¹⁴. Little or no nitrite formation occurred in non-irradiated samples maintained at the same temperature or in irradiated samples in which Wylde Lake water was replaced by distilled water.

production was initially ~5 μmol l⁻¹ h⁻¹ and declined slightly with time. SOD produced a substantial inhibition of the reaction; this inhibitory effect was largely but not completely lost if the enzyme solution was boiled. The small residual inhibition was possibly due to Cu²⁺ ion, which forms part of the bovine erythrocyte SOD molecule and is itself effective in catalysing superoxide disproportionation.

As far as is known, SOD only catalyses the dismutation of superoxide; earlier suggestions that it might act as a quencher of singlet oxygen are now believed to have been mistaken¹². It is difficult, therefore, to interpret our results in any way other than as demonstrating the photochemical production of superoxide.

The mechanism of the reaction, however, is unclear. Although oxygen is consumed in the photochemical iron oxidation–reduction cycle⁴, generation of superoxide in this process has not been reported. An appropriate model for this cycle appears to be iron EDTA since iron (II) produced as a result of photodecarboxylation of the ligand is rapidly reoxidized to iron(III) by dissolved oxygen¹⁵. We found no trace of nitrite production on irradiation of a solution of 0.05 M sodium EDTA and 10⁻⁴ M hydroxylamine with 0.01 M total iron at pH 7.4 and therefore consider it unlikely that the iron redox cycle is involved in the generation of superoxide.

A possible model for superoxide production is provided by sodium anthraquinone-2-sulphonate (AQS). AQS in the excited state can react with any of a variety of electron donors to produce the AQS semiquinone radical anion (AQS^{•-}). Another molecule of AQS can serve as the electron donor and is converted to a yellow hydroxylated AQS derivative^{16,17}. The semiquinone radical ion then reacts with oxygen:



The formation of superoxide ion by the autoxidation of semiquinones has been reported elsewhere^{18,19}.

Irradiation of a solution of 5 × 10⁻⁵ M AQS and 10⁻³ M hydroxylamine in phosphate buffer resulted in the rapid production of nitrite (initial rate ~25 × 10⁻⁵ mol l⁻¹ h⁻¹). The solution

became yellow in colour. The rate of nitrite production was reduced by two-thirds in the presence of $5 \mu\text{g ml}^{-1}$ SOD.

As a variety of quinone and semiquinone structures have been found to occur in the humic materials of soil²⁰ and are therefore probably also present in the very similar material making up aquatic humus, the above model appears reasonable.

We think it possible that photochemically produced superoxide may contribute to the degradation of certain environmental pollutants. Zepp *et al.*²¹ found that various natural water samples sensitized a photochemical dechlorination of methoxychlor by a mechanism not involving singlet oxygen. Superoxide has been shown to mediate reactions of this kind²².

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Predicting Indian monsoon rainfall from sea-surface temperature in the Indonesia–north Australia area

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Several recent studies^{1–6} have described associations between inter-annual variations of the Indian south-west monsoon and the El Niño–Southern Oscillation (ENSO) phenomena, and methods for predicting monsoon rainfall based on these associations have been proposed. Rasmusson and Carpenter⁵ suggested that sea-surface temperature (SST) anomalies appearing along the equatorial Pacific South American coast early in the year can provide useful predictions of Indian monsoon rainfall, while Shukla⁶ suggested that the change in atmospheric pressure anomalies at Darwin from winter to spring (Northern Hemisphere seasons) was useful. It has also been demonstrated^{8–11} that SST anomalies in the Indonesia–north Australia region are closely related to the ENSO phenomena. I examine here the relationship between Indian south-west monsoon rainfall and Indonesian–north Australian SST and provide evidence suggesting that SST anomalies in this region can be used to predict Indian monsoon rainfall as accurately as the techniques proposed previously, but using data available several months earlier.

The Southern Oscillation (SO) is a broad-scale fluctuation, on an inter-annual time scale, of the atmospheric and oceanic circulation. Fluctuations in the SO are associated with variations in surface pressure, low and upper-level winds, rainfall, and SSTs from their normal seasonal patterns, especially over the tropical Pacific Ocean. (Philander⁷ has recently summarized the phenomenon.) Also associated with the SO are occurrences of El Niño, an anomalous warming of the usually cool

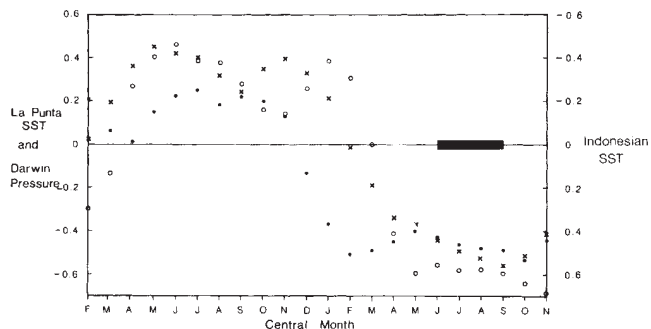


Fig. 1 Correlations between a rainfall index for the Indian monsoon from June to September with three-month means of sea-surface-temperatures in the Indonesian region averaged over the area 120°E – 160°E 5°S – 15°S (●), with Darwin pressures (○), and with sea-surface-temperatures at La Punta/Callao on the Peruvian coast (×). Data from 1964–79. Period of Indian monsoon indicated by thick horizontal bar. Central month of three-month periods indicated on abscissa. Note the reversal of scale for the correlations with Indonesian SST.

waters of the east equatorial Pacific that generally occurs at intervals of several years.

Recent studies^{1–4} have demonstrated the existence of a relationship between El Niño events and Indian monsoon rainfall, with rainfall tending to be deficient in El Niño years. Rasmusson and Carpenter⁵ suggested that this relationship might provide a means for forecasting Indian south-west monsoon rainfall. The rationale for this proposal was that El Niño anomalies, if they are going to occur in a particular year, usually appear before the start of the Indian monsoon (June–September). Thus the appearance or non-appearance of the El Niño could be used to predict Indian monsoon rainfall. Shukla⁶ noted that a relationship also exists between Indian monsoon rainfall and the change in Darwin pressure (an index of the SO) from the Northern Hemisphere winter to spring. When pressure anomalies decreased over this period drought was unlikely to occur over India. Again, the rationale was that the changes in Darwin pressure tended to occur before the start of the south-west monsoon.

It also has been shown that SST anomalies in the Indonesian–north Australian region are related to the ENSO phenomena. Newell *et al.*⁸ found significant and seasonally varying correlations between Indonesian SST and the SO. Rasmusson and Carpenter⁹ composited Pacific anomalies in seasons near El Niño occurrences. Their study also indicates that Indonesian SST anomalies are related to El Niño and that changes in the Indonesian SST anomalies precede changes in east equatorial Pacific SST anomalies by several months (their Figs 17–22). I have described^{10,11} the relationship between Indonesian SST and ENSO in more detail and postulated a possible cause of this relationship.

The relationship of Indonesian SSTs to ENSO and the tendency for them to lead east Pacific SSTs suggests that they might provide forecasts of Indian monsoon rainfall with longer lead times than is possible using either Darwin pressure tendencies or east Pacific SSTs. This study correlates three-monthly means of SST averaged over the region 120°E – 160°E , 5°S – 15°S , with an index of area-averaged June–September rainfall over India. Before 1964 the SST data are poor so only data after this date are used. The Indian rainfall index was taken from ref. 5 and was only available up to 1979. Therefore only 1964–79 data were used in this study. Correlations between the Indian rainfall index and Indonesian SST and also with SST at La Punta/Callao (12°S , 77°W) on the Pacific coast of South America, and with Darwin (12°S , 131°E) air pressure were calculated. The results are shown in Fig. 1 which plots the correlations for the three independent variables against the central month of the three-month averaging period. Note that the scale for the Indonesian SSTs is reversed. Correlations of Indian monsoon rainfall with

values of the three variables up to 16 months earlier (that is in the previous year) are shown.

Figure 1 demonstrates that Indian monsoon rainfall is correlated, simultaneously, negatively with east Pacific SST and Darwin pressure, and positively with Indonesian SST. Correlations of opposite sign are found with values of the three independent variables in the year before that of the monsoon. A tendency for the sign of the correlations with each of the three independent variables to change sign some time before the start of the monsoon is also evident in Fig. 1. The correlations with east Pacific SST anomalies change from positive to negative around February, confirming the suggestion of Rasmusson and Carpenter, but do not reach statistically significant magnitudes (5% significance level is 0.51) until around August during the monsoon period. The Darwin pressure correlations change sign around March, as shown by Shukla, and reach the 5% significance level in May. As was noted by Rasmusson and Carpenter and by Shukla, changes in east Pacific SST and Darwin pressure anomalies from negative to positive around February or March suggest that the following Indian south-west monsoon will be drier than usual.

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Reduced advection into Atlantic Ocean deep eastern basins during last glaciati on maximum

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Causes of change in deep water $\delta^{13}\text{C}$ can be either global or local in extent. Global causes include (1) climatically-induced changes in the amount of terrestrial biomass which alter the average carbon isotopic composition of the oceanic reservoir¹, and (2) erosion and deposition of organic-rich, continental shelf sediments during sea level fluctuations which change the mean oceanic carbon:phosphorus ratio². Regional gradients of $\delta^{13}\text{C}$ are created by remineralization of organic detritus within the deep ocean itself thus reflecting the distribution of water masses and modern thermohaline flow. Changes in a single geological record of benthic foraminiferal $\delta^{13}\text{C}$ can result from any combination of these global and abyssal circulation effects. By sampling a large number of cores collected over a wide bathymetric range yet confined to a small geographical region we have minimized the ambiguity. We can assume that each $\delta^{13}\text{C}$ record was equally affected by global causes of $\delta^{13}\text{C}$ variation. The differences seen between the $\delta^{13}\text{C}$ records must, therefore, reflect changes in the distribution of ^{13}C in the deep ocean. We interpret these differences in distribution in terms of changes in the ocean's abyssal circulation. Benthic foraminiferal carbon isotopic evidence from a suite of Sierra Leone Rise cores indicates that the deeper parts of the eastern Atlantic basins underwent a reduction in $[\text{O}_2]$ during the maximum of the last glaciati on. Reduced advection of O_2 -rich deep water through low-latitude fracture zones, associated with increased delivery of organic matter to the deep ocean, lowered the $\delta^{13}\text{C}$ of deep water ΣCO_2 at all depths below the sill separating the eastern and western Atlantic basins.³ This decreased advection into the eastern Atlantic Ocean coincides with the overall decrease in deep water production in the North Atlantic during the last glaciati on maximum^{4–7}.

Nine giant gravity cores⁸ (GGC; diameter = 10 cm) were selected from among 42 cores recovered in 1981 from the slopes of the Sierra Leone Rise by RV *Endeavor* cruise 66. These nine

The correlations between Indonesian SST and Indian monsoon rainfall change sign rather earlier, around November or December in the year before the monsoon. A dry Indian monsoon would be foreshadowed by a decrease in SST anomalies in the Indonesian region around this time. The correlations reach the 5% significance level around February. Thus the precursors of Indian monsoon rain in Indonesian SST lead those in east Pacific SST and Darwin pressure by several months, providing a longer lead time for predicting Indian monsoon rainfall. The correlations for the three independent variables are comparable in magnitude suggesting that the increase in forecast lead-time obtainable from the use of Indonesian SSTs is not gained at the expense of forecast accuracy.

The amount of data used to produce Fig. 1 are small and thus these conclusions must remain tentative until further data become available. The results do suggest, however, that the routine and careful monitoring of SSTs in the Indonesian–north Australian region could be worthwhile in the context of developing an accurate method for the long-range prediction of the Indian monsoon.

SSTs were provided by P. B. Wright.

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cores range from water depths between 2,931 m and 5,104 m (see Table 1). Their stratigraphical integrity was documented with $\delta^{18}\text{O}$, high resolution per cent carbonate analysis and biostratigraphy (correlated occurrences of the planktonic foraminifer *Globorotalia menardii*⁹ and the diatom *Ethmodiscus rex*¹⁰). Within the resolution of these stratigraphical techniques, sediment accumulation in each core is continuous at rates between 1.5 and 2.7 cm kyr⁻¹. Oxygen and carbon isotopic compositions were analysed on 5–10 individual calcitic tests of the benthic foraminiferal species *Planulina wuellerstorfi*, a species whose $\delta^{13}\text{C}$ variations have been shown to reflect accurately the modern distribution of ^{13}C in the oceans^{11,12}. Standard analytical procedures were followed^{4,13}, and analytical precision is better than $\pm 0.1\%$ for measurements of both $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$.

The nine records of past carbon isotopic changes are presented in Fig. 1. Glacial–interglacial differences in $\delta^{13}\text{C}$ for each core shallower than 3,750 m (the sill depth separating eastern and western equatorial Atlantic basins) are nearly equal in both timing and magnitude. The change in $\delta^{13}\text{C}$ at the last deglaciation (age 13,000 yr, ref. 13) is $\sim 0.6\%$ in the four shallowest cores. In contrast, the $\delta^{13}\text{C}$ amplitude at the deglaciation increases in cores recovered from below the sill depth. In the deepest core, $\delta^{13}\text{C}$ values for *P. wuellerstorfi* during the interval 13,000–32,000 yr (glacial isotope stage 2) are $\sim 0.7\%$ lower than contemporaneous benthic foraminiferal $\delta^{13}\text{C}$ in cores recovered from shallower than the sill depth. The amplitude of $\delta^{13}\text{C}$ at the deglaciation is ~ 1.2 – 1.3% in the two deepest cores.

Figure 2 shows the modern bathymetric profile of $\delta^{13}\text{C}$ of ΣCO_2 in deep water in the eastern equatorial Atlantic¹⁴ and the associated profile of *P. wuellerstorfi* $\delta^{13}\text{C}$ in both Holocene samples and samples from glacial isotope stage 2. Neither the $\delta^{13}\text{C}$ values of *P. wuellerstorfi* in Holocene samples nor the $\delta^{13}\text{C}$ values measured in the water column exhibit any significant gradient with increasing water depth. In contrast, the $\delta^{13}\text{C}$ bathymetric profile reconstructed from glacial samples displays a large decrease with depth. In the modern ocean, a gradient in $\delta^{13}\text{C}$ of this magnitude is equivalent to an apparent oxygen utilization (AOU) gradient of ~ 90 – $100\ \mu\text{mol kg}^{-1}$ (ref. 15). The deep water in the deepest part of the basin was reduced in $[\text{O}_2]$ relative to water shallower than the sill. This steepened gradient may have resulted from increased supply of organic carbon or decreased supply of O_2 to the deep basin.

At present, the flux of deep water through low-latitude fracture zones is the main source of oxygen to the eastern basins³.

Table 1 Oxygen and carbon isotopic composition of *P. wuellerstorfi* in cores from the Sierra Leone Rise, eastern equatorial Atlantic Ocean

Depth (m)	Age (kyr)	$\delta^{18}\text{O}_{\text{PDB}}$	$\delta^{13}\text{C}_{\text{PDB}}$	Depth (m)	Age (kyr)	$\delta^{18}\text{O}_{\text{PDB}}$	$\delta^{13}\text{C}_{\text{PDB}}$	Depth (m)	Age (kyr)	$\delta^{18}\text{O}_{\text{PDB}}$	$\delta^{13}\text{C}_{\text{PDB}}$
EN066-10GGC 6.65°N 21.90°W 3,527(m)				EN066-26GGC 3.09°N 20.02°W 4,745(m)				EN066-36GGC 4.31°N 20.21°W 4,270(m)			
0.04	2.7	2.25	0.82	0.04	2.7	2.64	0.84	0.04	3.1	2.59	0.99
0.07	4.8	2.49	0.80	0.10	6.8	2.67	0.52	0.04	3.1	2.91	0.51
0.10	6.8	2.69	0.85	0.14	9.6	3.04	0.24	0.07	5.4	2.87	0.63
0.14	9.6	3.21	0.48	0.17	11.6	3.28	0.51	0.10	7.6	2.99	0.60
0.17	11.6	2.92	0.55	0.20	13.7	3.62	0.20	0.14	10.7	3.56	0.58
0.24	18.0	3.96	0.53	0.24	16.7	4.19	0.19	0.17	13.0	3.12	0.75
0.27	21.0	4.02	0.40	0.27	18.8	4.11	0.10	0.20	15.0	3.75	0.33
0.30	24.0	3.87	0.36	0.30	21.0	4.16	-0.16	0.24	17.8	4.28	0.38
0.34	28.0	4.11	0.19	0.34	24.0	4.23	-0.18	0.27	19.8	4.24	0.19
0.37	31.0	3.91	0.47	0.37	26.2	4.03	-0.25	0.30	21.8	4.18	0.06
0.40	33.5	3.81	0.54	0.40	28.3	4.10	-0.06	0.34	24.5	4.29	0.23
0.44	36.5	3.79	0.44	0.44	31.3	4.23	0.10	0.34	24.5	4.33	0.16
0.47	38.7	3.79	0.54	0.47	33.2	4.01	0.05	0.37	26.6	4.27	0.02
0.47	38.7	3.89	0.48	0.50	35.1	3.97	0.13	0.44	31.3	3.95	0.22
0.50	40.9	3.79	0.65	0.60	41.2	3.59	0.07	0.47	33.0	3.95	0.14
0.60	48.4	3.56	0.50	0.70	47.4	3.56	0.12	0.60	39.9	3.70	0.17
								0.70	45.1	3.54	0.04
								0.70	45.1	3.69	0.13
								0.80	50.4	3.81	0.32
								0.80	50.4	3.78	0.35
EN066-16GGC 5.46°N 21.14°W 3,152 (m)				EN066-29GGC 2.46°N 19.76°W 5,104(m)				EN066-38GGC 4.92°N 20.50°W 2,931(m)			
0.04	2.9	2.54	0.94	0.04	2.4	2.41	0.85	0.03	2.2	2.52	0.98
0.07	5.1	2.44	0.99	0.04	2.4	2.72	0.84	0.03	2.2	2.75	1.11
0.10	7.2	3.35	0.57	0.10	5.9	2.86	0.48	0.06	4.3	2.69	1.24
0.14	10.1	2.81	0.95	0.14	8.2	3.01	0.77	0.09	6.5	3.17	0.93
0.17	12.3	3.09	0.77	0.20	11.8	3.14	0.21	0.09	6.5	2.82	0.85
0.20	14.1	4.14	0.47	0.20	11.8	3.19	0.18	0.12	8.7	2.96	1.09
0.24	16.4	4.38	0.44	0.24	14.1	4.03	-0.04	0.16	11.6	3.17	0.69
0.27	18.0	4.19	0.31	0.30	17.3	3.87	-0.29	0.19	14.0	3.99	0.41
0.30	19.7	4.35	0.43	0.30	17.3	4.24	-0.34	0.22	17.0	4.05	0.50
0.34	21.9	4.38	0.43	0.40	22.8	3.50	-0.16	0.26	21.0	4.23	0.69
0.37	23.6	4.30	0.40	0.50	28.2	4.00	-0.23	0.29	24.0	3.98	0.59
0.40	25.3	4.08	0.46	0.54	30.4	3.99	0.01	0.32	27.0	3.93	0.41
0.44	27.5	4.13	0.51	0.60	33.1	3.62	0.27	0.35	30.0	4.00	0.52
0.44	27.5	4.15	0.50	0.64	34.6	3.44	0.01	0.39	33.3	3.94	0.55
0.47	29.2	4.20	0.54	0.70	36.9	3.75	0.06	0.39	33.3	3.90	0.60
0.50	30.9	4.14	0.44					0.42	35.1	3.85	0.62
0.50	30.9	3.96	0.28					0.46	37.6	3.89	0.70
0.60	36.4	4.02	0.71					0.49	39.5	3.92	0.53
0.70	41.9	3.55	0.84					0.69	52.1	3.70	0.79
0.70	41.9	3.97	0.81								
0.80	47.4	3.73	0.69								
EN066-21GGC 4.23°N 20.63°W 3,995(m)				EN066-32GGC 2.47°N 19.74°W 5,003(m)				EN066-44GGC 5.26°N 21.71°W 3,428(m)			
0.03	1.8	2.50	0.78	0.04	2.4	2.76	0.86	0.04	4.3	2.62	1.15
0.06	3.5	2.54	0.83	0.07	4.1	2.68	0.91	0.04	4.3	2.55	0.94
0.11	6.5	2.55	0.90	0.10	5.9	2.88	0.91	0.07	7.6	2.95	0.95
0.16	9.5	3.06	0.69	0.14	8.3	3.18	0.60	0.07	7.6	2.90	1.15
0.20	11.8	3.42	0.54	0.17	10.0	2.93	0.82	0.10	10.8	3.69	0.61
0.23	14.0	3.53	0.34	0.20	11.8	3.31	0.54	0.14	14.8	3.59	0.59
0.26	16.8	3.88	0.17	0.24	14.0	3.46	0.63	0.17	17.5	4.40	0.60
0.30	20.6	4.09	0.12	0.30	16.8	4.11	0.41	0.20	20.2	4.31	0.54
0.33	23.5	4.07	0.20	0.34	18.7	4.09	0.04	0.20	20.2	3.87	0.40
0.36	26.3	3.72	0.02	0.37	20.1	4.08	0.06	0.24	23.9	4.36	0.45
0.40	30.1	4.03	0.26	0.40	21.6	4.05	-0.30	0.27	26.6	4.60	0.62
0.43	32.5	3.98	0.28	0.64	32.8	3.89	-0.19	0.27	26.6	3.94	0.48
0.46	34.2	3.97	0.42	0.70	35.3	3.80	0.22	0.30	29.3	4.71	0.48
0.50	36.3	3.84	0.39	0.74	36.9	3.73	0.14	0.30	29.3	4.16	0.50
0.56	39.6	3.41	0.08	0.77	38.2	3.74	0.17	0.34	32.7	4.65	0.75
0.66	45.0	3.64	0.54					0.34	32.7	4.02	0.55
0.66	45.0	3.82	0.42					0.37	34.8	4.14	0.61
0.76	50.4	3.52	0.39					0.37	34.8	4.01	0.57
0.76	50.4	3.97	0.39					0.37	34.8	3.81	0.53
								0.40	37.0	4.04	0.69
								0.44	39.8	4.17	0.62
								0.47	42.0	3.94	0.71
								0.47	42.0	3.97	0.84
								0.47	42.0	3.82	0.67
								0.50	44.1	4.11	0.75
								0.60	51.2	3.40	0.44

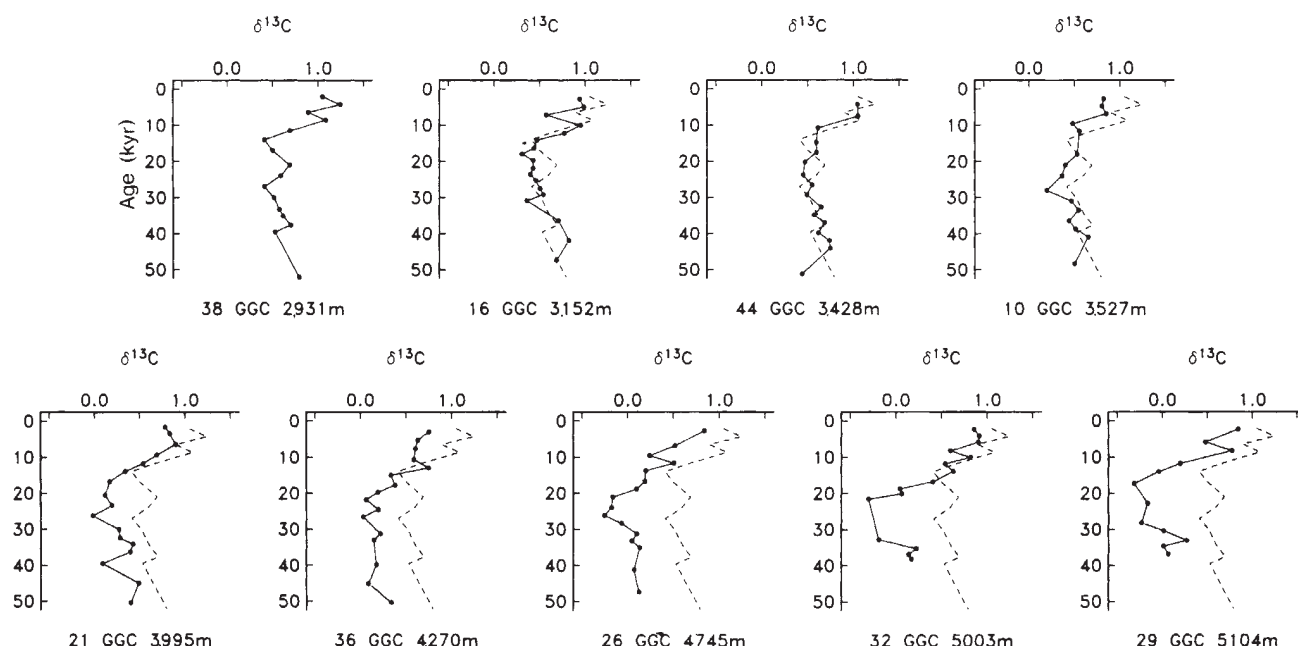


Fig. 1 Carbon isotopic data for nine gravity cores from the Sierra Leone Rise. From $\delta^{18}\text{O}$ and CaCO_3 records, we have identified the stage 1/2 and 2/3 boundaries in each core and assigned to them ages of 13,000 and 32,000 yr respectively¹³. The four cores in the upper row are located at water depths shallower than the sill (3,750 m) separating the eastern and western basins of the Atlantic Ocean. The carbon isotopic record from the shallowest core (38GCC, 2,931 m) is plotted (dashed line) as a reference on each of the other records. None of the cores shallower than the sill exhibits any significant difference from this reference $\delta^{13}\text{C}$ record. Each of the cores located at water depths deeper than the sill diverge from the reference record during the last glacial maximum. The magnitude of the divergence increases with depth. The maximum difference between the reference record and the deepest $\delta^{13}\text{C}$ record (29GGC, 5,104 m) is 0.7‰.

The eastern basins are bounded to the west by the Mid-Atlantic Ridge and, to the south, the Walvis Ridge is an effective barrier against influx of well-oxygenated water. Two fracture zones, the Romanche and Vema, are potential conduits of deep water to the eastern basins¹⁶, although Metcalf *et al.*³ have concluded that the Romanche Fracture Zone is the principal conduit. They noted that depth profiles of potential temperature from western and eastern basin locations diverge at ~3,750 m and concluded that this is the effective sill depth of the Mid-Atlantic Ridge. Shallower than this depth, communication between the eastern and western Atlantic appears to be unrestricted while deeper than this depth, topographical barriers restrict flow. A north-south cross-section¹⁷ of dissolved oxygen concentration in the eastern Atlantic Ocean shows the presence of well-oxygenated water at ~4,000 m at the Equator. To the north and south, dissolved O_2 concentrations decrease. Although carbon isotopic analyses of ΣCO_2 have been undertaken for only two hydrographic stations within the eastern basins (GEOSECS stations 113 and 107), these limited data do not show large latitudinal or bathymetric gradients. Within the basin, $\delta^{13}\text{C}$ is constant at $\sim 0.8 \pm 0.1\%$.

The AOU difference between the western basin at the sill depth and the eastern basin below the sill depth is determined by the balance of organic matter oxidation within the eastern basins and advection of O_2 across the Romanche Fracture Zone. Although the processes involved are complex, a simplified model can be presented to illustrate some of the important factors controlling the AOU (and $\delta^{13}\text{C}$) difference between the eastern and western Atlantic. Assuming steady state, the relationship is

$$\Delta\text{AOU} = \frac{C_{\text{flux}}\text{SO}_2/\text{C}}{A} \quad (1)$$

where ΔAOU is the AOU difference between deep water at 3,750 m in the western basin and below 3,750 m in the eastern basin (in mol m^{-3}); C_{flux} is the flux of organic carbon into the eastern basin which is being remineralized (in $\text{mol m}^{-2} \text{s}^{-1}$); S is the surface area of the eastern basin deeper than 3,750 m ($\sim 1.8 \times 10^{13} \text{ m}^2$); O_2/C is the ratio of O_2 consumption and organic carbon oxidation during the oxidation process, an

empirical relationship¹⁸ assumed to have remained constant at a value of 138/105; A is the advection of water across the Romanche Fracture Zone ($\text{m}^3 \text{s}^{-1}$).

The AOU difference between the eastern and western basins near the Equator in the present Atlantic Ocean is $\sim 15 \mu\text{mol kg}^{-1}$ ($10^{-3} \text{ mol m}^{-3}$) (ref. 17). Using this value and assuming reasonable rates for organic matter oxidation within the basin, it is possible to calculate the rate of advection into the basin below the sill. For example, given that $\Delta\text{AOU} = 15 \mu\text{mol kg}^{-1}$ and assuming a $C_{\text{flux}} = 2 \text{ mg m}^{-2} \text{ day}^{-1}$ (or $1.9 \times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$, a typical rate of organic carbon decay in deep water¹⁹), the advection of deep water into the eastern basin below the sill must be $\sim 3 \times 10^6 \text{ m}^3 \text{ s}^{-1}$. If the amount of organic carbon oxidation within the eastern Atlantic is larger, the advective term in equation (1) must also be larger to maintain the present ΔAOU value. The ΔAOU between the basins is a direct function of the amount of organic matter oxidation in the basin and a reciprocal function of the advection into the basin. The ΔAOU value is independent of the initial AOU value of the deep water entering the eastern Atlantic.

The vertical profile of $\delta^{13}\text{C}$ in Fig. 2 represents a ΔAOU value of $90\text{--}100 \mu\text{mol kg}^{-1}$ above and below the sill during the glacial maximum; this is a minimum estimate of the ΔAOU value between the western and eastern basins. An increase in $[\text{O}_2]$ from west to east above the sill would imply an oxygen source in the eastern basins which, while not impossible, is unlikely. It is more likely that well-oxygenated water masses be found in the western basin and that ΔAOU would increase ($[\text{O}_2]$ would decrease) from the western into the eastern basins. In this case, the vertical AOU at the Sierra Leone Rise represents the minimum ΔAOU expected across the fracture zone. This vertical gradient of AOU then places boundary conditions on the balance of organic matter input and advection into the eastern basins.

If the glacial profile of AOU is a reasonable estimate of ΔAOU between the eastern and western basins, it represents a sixfold increase over the modern observed ΔAOU of $\sim 15 \mu\text{mol kg}^{-1}$. From equation (1), this must be the result of: (1) a sixfold increase in the oxidation of organic carbon in the

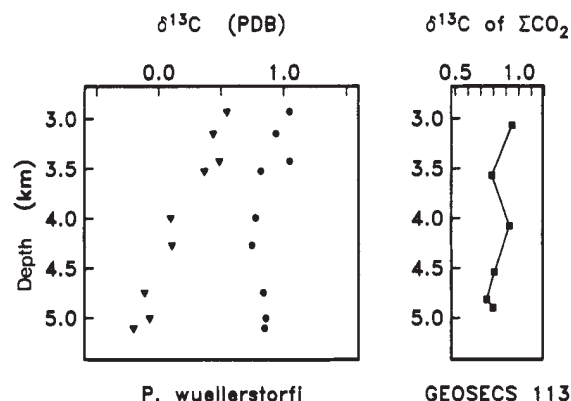


Fig. 2 Profiles of $\delta^{13}\text{C}$ measured on the ΣCO_2 of the modern water column¹⁴ (■), on Holocene core top *P. wuellerstorfi* (●), and glacial (^{18}O stage 2) *P. wuellerstorfi* (▼). The glacial values for *P. wuellerstorfi* are averages of 18,000–30,000-yr old samples in each core. While there is little significant change in $\delta^{13}\text{C}$ with depth in either the modern water column or modern benthic foraminifera, the glacial benthic foraminifera exhibit a decrease of $\sim 0.7\%$ with increasing depth. This vertical gradient is an estimate of the $\delta^{13}\text{C}$ difference between the western basin at the sill depth and the eastern basin below the sill.

deeper parts of the basin with no change in circulation; (2) a sixfold decrease in the advection into the deeper parts of the basin with no change in primary production; or (3) some combination of these two factors.

It is unlikely that the AOU gradient that developed during the glacial maximum resulted solely from an increase in the amount of organic carbon oxidation occurring below the sill. Other investigations have determined that, locally, productivity during stage 2 may have increased by a factor of 2 or 3 (refs 20, 21); these studies, however, have emphasized coastal or equatorial upwelling regions. If these observations are correct, the increase in ΔAOU must have resulted, at least in part, from a reduction in the advection of deep water into the basin. Given a conservative estimate of a two- to threefold increase in the rain of carbon into the basin, the advective term in equation (1) must have been reduced by a factor of two. This reduced advection into the eastern basin probably resulted from the overall decrease in deep water production thought to have occurred during the last glacial maximum^{4–7}.

It is clear that the advection of deep water into the basin did not stop completely. If it had, under reasonable rates of carbon input, all dissolved oxygen in the basin below sill depth would have been consumed in a few thousand years. The presence of fossilized aerobic benthic organisms in the glacially deposited sediments indicates that this did not happen. Some flux of O_2 into the basin must have occurred during the last glacial maximum.

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Retene—a molecular marker of wood combustion in ambient air

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The use of wood as a fuel has increased since the oil embargo in 1973. Several studies have shown that wood combustion may make a significant contribution to air pollution. Using ^{14}C as a tracer for contemporary carbonaceous materials, 30–70% of the atmospheric carbon has been shown to originate from wood combustion in areas affected by this source^{1–3}. Other studies have shown that emissions from wood combustion contain large amounts of particles^{4–6} and organic compounds, one class being polycyclic aromatic hydrocarbons (PAH)^{7–11}. However, these compounds are also formed by combustion of other carbonaceous materials. In our studies on PAH in wood combustion emissions and in ambient air in wood-heated residential areas, we have identified several PAH compounds which may be related to combustion of coniferous wood. These are alkylated phenanthrene compounds with the main compound 1-methyl-7-isopropylphenanthrene (trivial name retene) formed by thermal degradation of resin compounds in the wood.

Samples from wood combustion were taken from a chimney stack using a quartz glass probe. The particles in the flue gas were sampled on Gelman AE glass fibre filters held at 125 °C. The flue gas was cooled and the condensate collected in several impingers cooled gradually from 0 to –60 °C. Volatile organics in the flue gas were then adsorbed on a 5 × 5 cm column containing Amberlite XAD-2. Samples were taken from a small 4–5 kW horizontal baffled stove and a 50 kW hot water boiler. Spruce (*Picea abies*) was used as fuel in both units, birch was also burned in the stove as a reference fuel for hardwoods.

Samples of ambient air were taken with Sierra high-volume samplers by members of the Norwegian Institute for Air Research in Elverum, Norway³. The samples were collected for 24 h and consisted of about 1,600 m³ air. A cascade impaction stage ensured that only particles <3 µm in diameter were collected. Wood combustion has been estimated by use of ^{14}C as a tracer for contemporary carbon³, to contribute ~65% of the total carbon in ambient fine particles in the Elverum ambient air, and the city, which has 10,000 inhabitants, is therefore well suited for studying ambient impact of wood combustion.

The chromatographic profile of the PAH fraction in a spruce combustion sample from the hot water boiler is shown in Fig. 1a. The main components in the chromatogram originate more or less from combustion of any carbonaceous material with the exception of peak 23 which is not present in other combustion samples from birch^{9,11}, straw¹², peat⁹, coal^{10,13}, oil¹⁰ or petrol¹⁴. In emission samples, this compound seems, therefore, to originate only from combustion of spruce and related resinous softwoods. The compound has been identified by gas chromatography-mass spectrometry (GC-MS) using a Finnigan 4021 instrument and by GC retention time comparison with an authentic standard to be 1-methyl-7-isopropylphenanthrene with the trivial name retene. The retene in the combustion products of spruce is apparently derived from diterpenoid resin acid, abietic acid, which is present in the resins of conifers^{15–17}. Figure 2

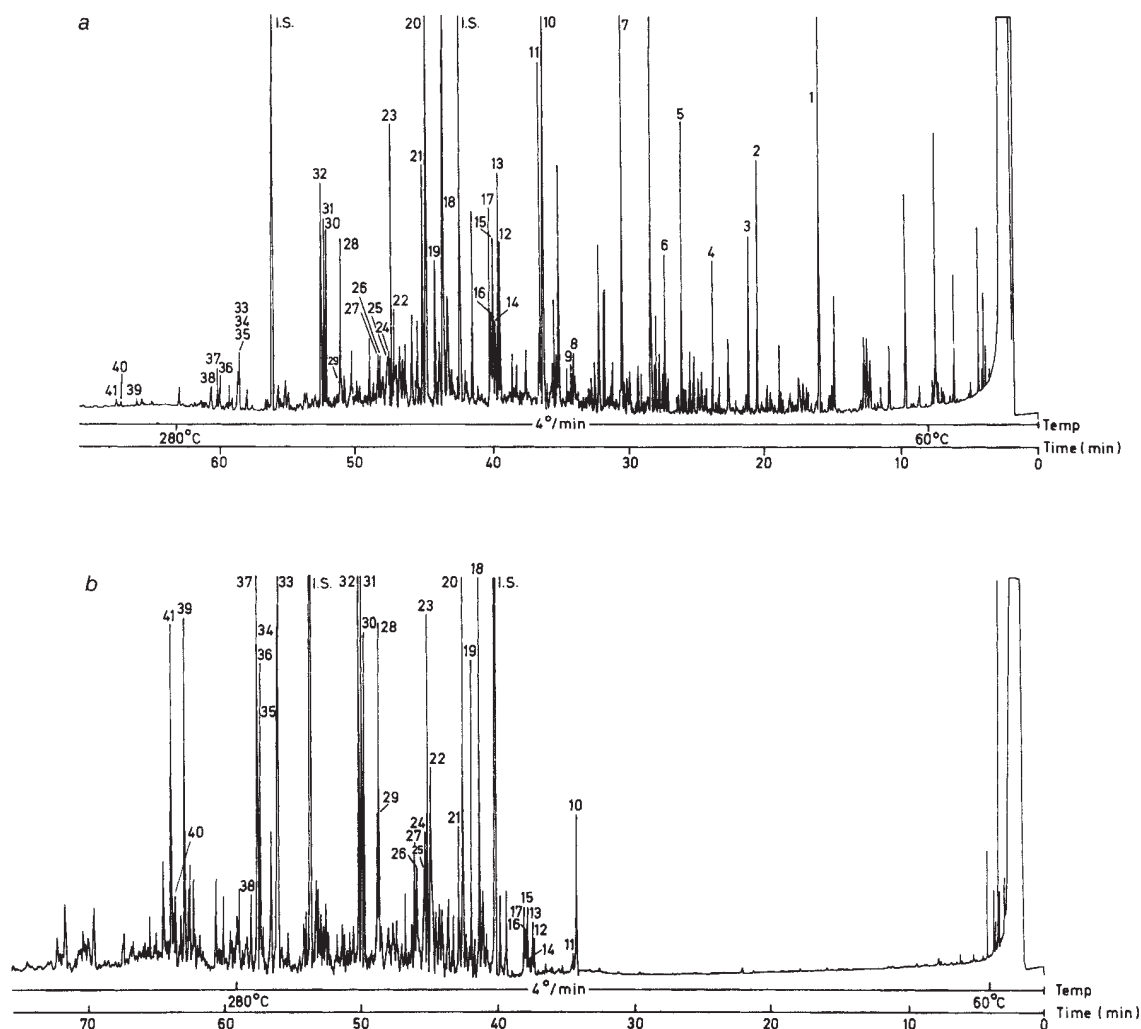


Fig. 1 Chromatographic profiles of the PAH fraction of *a*, a wood combustion emission sample from a 50-kW hot water boiler burning spruce, and *b*, an ambient air sample from Elverum, Norway, a city which uses a lot of wood for heating. The samples were extracted for 24 h in a Soxhlet apparatus with dichloromethane (DCM), except for the condensate from the emission measurements which was liquid/liquid extracted with DCM. The condensate extract was then added to the other emission extracts to give a total extract. The DCM extracts were taken to near dryness and cyclohexane were added. The resulting cyclohexane extracts were subjected to liquid/liquid partition with dimethylformamide²⁷. The analyses were performed with a Hewlett-Packard 5730A gas chromatograph equipped with a 50 m SE-54 fused silica capillary column and a flame ionization detector. Peak identities: 1, naphthalene; 2, 2-methylnaphthalene; 3, 1-methylnaphthalene; 4, bisphenyl; 5, acenaphthylene; 6, acenaphthene; 7, fluorene; 8, 2-methylfluorene; 9, 1-methylfluorene; 10, phenanthrene; 11, anthracene; 12, 3-methylphenanthrene; 13, 2-methylphenanthrene; 14, 2-methylanthracene; 15, 4,5-methylenepheneanthrene; 16, 9- and/or 4-methylphenanthrene; 17, 1-methylphenanthrene; 18, fluoranthene; 19, acephenanthrylene; 20, pyrene; 21, ethylmethylenepheneanthrene?; 22, benzo(*a*)fluorene; 23, 1-methyl-7-isopropylphenanthrene(retene); 24, benzo(*b*)fluorene; 25, 4-methylpyrene; 26, 2-methylpyrene; 27, 1-methylpyrene; 28, benzo(*ghi*)fluoranthene; 29, benzo(*c*)phenanthrene; 30, cyclopenta(*cd*)pyrene; 31, benz(*a*)anthracene; 32, chrysene and triphenylene; 33, benzo(*b*)fluoranthene; 34, benzo(*j*)fluoranthene; 35, benzo(*k*)fluoranthene; 36, benzo(*e*)pyrene; 37, benzo(*a*)pyrene; 38, perylene; 39, indeno(1,2,3-*cd*)pyrene; 40, dibenz(*a,c* and/or *a,h*)anthracenes; 41, benzo(*ghi*)perylene; IS, internal standards.

shows the hypothetical thermal degradation of abietic acid (I) to retene (IV). The presence of abietic acid derivatives in wood combustion emissions has been noted by several workers^{18–20}, and a degradation scheme similar to that shown in Fig. 2 is supported by the mass spectral identification of several tricyclic aromatic compounds in the emissions: 19-norabieta-8,11,13-triene, 19-norabieta-4,8,11,13-tetraene, dehydroabietin and simonellite with mass spectra identical to those reported by Simoneit and Mazurek²¹. Pereira *et al.* observed several alkylated phenanthrene derivatives in volcanic ash from the Mt St Helens 1980 volcanic eruption and related these to pyrolysis of plant material²⁰.

Table 1 gives the amount of retene and other main PAH compounds in the emission samples. The yield of retene from the thermal degradation of abietic acid seems to be very dependent on temperature and air supply. When burning spruce in starved air conditions, as in sample 1 in Table 1, the temperature

was 260 °C in the stove and the flue gas temperature was only 45 °C. This resulted in retene being the most important PAH formed. With a richer air supply and higher combustion temperatures as shown in the other emission samples the relative amount of retene formed is less, but still very significant. This demonstrates that the formation mechanism for retene is different from that of the other PAH which is believed to involve cracking of organic compounds to smaller, unstable molecules. These fragments, mostly radicals, recombine to larger aromatic ring systems by pyrosynthesis²². When the temperature gets higher the abietic acid will be more susceptible to cracking and hence the yield of retene will be lower.

As shown in Fig. 1*b* retene is also an important peak in the chromatogram of the PAH fraction of the Elverum ambient air, demonstrating that the retene released from wood combustion makes a significant contribution to the total PAH in the ambient air of the city. Note that spruce only amounts to about one-

Table 1 Retene and other polycyclic aromatic hydrocarbons in samples related to wood combustion

Peak no.	Compound	Sample no. Sample from	1 4–5 kW stove*	2 3.1 kg h ⁻¹ μg m ⁻³	3 50 kW boiler*	4 40 kg h ⁻¹ flue gas	5	6 Elverum ambient air†	7	8
		Burning rate Concentration	0.4 kg h ⁻¹	3.1 kg h ⁻¹ μg m ⁻³	40 kg h ⁻¹	40 kg h ⁻¹	ng m ⁻³ air			
10	Phenanthrene		680	83	4,980	3,200	2.62	3.40	3.26	3.42
11	Anthracene		151	12	770	370	0.26	0.33	0.21	0.28
18	Fluoranthene		263	29	2,070	1,930	20.69	18.87	25.67	24.47
20	Pyrene		310	23	1,510	1,210	20.24	17.90	26.55	24.80
22	Benzo(a)fluorene		68	2	360	120	4.67	2.58	2.05	6.74
23	Retene		1,113	16	1,420	360	8.07	6.07	6.64	8.13
24	Benzo(b)fluorene	‡	1	190	110	3.18	1.92	2.59	3.30	
28	Benzo(ghi)fluoranthene		39	5	135	180	4.71	2.53	5.83	3.18
30	Cyclopenta(cd)pyrene		58	4	230	210	5.26	1.92	4.45	11.57
31	Benz(a)anthracene		63	6	250	240	7.56	3.89	7.90	15.38
32	Chrysene and Triphenylene	‡	67	520	320	9.31	5.50	13.44	8.19	
33–35	Benzo(b, j & k)fluoranthenes		82	11	270	100	14.45	8.67	11.03	1.51
36	Benzo(e)pyrene		28	4	80	50	5.83	2.45	3.69	5.14
37	Benzo(a)pyrene		50	6	110	60	6.29	2.91	4.73	6.39
38	Perylene		3	2	10	5	1.34	0.19	0.99	0.74
39	Indeno(1,2,3-cd)pyrene		24	3	40	15	5.54	2.40	3.95	5.17
41	Benzo(ghi)perylene		24	3	40	15	7.39	3.37	5.79	6.94
	Total		3,023	210	12,985	8,495	127.41	84.90	128.77	135.35

* Spruce (*Picea abies*) was used as fuel.

† The samples were taken between 19 and 23 February 1982 with a cold weather average temperature of -15°C (ref. 3).

‡ Hidden by an interfering compound.

quarter of the wood used in Elverum, the remaining fuelwood is mostly birch³. The analytical results of some samples from Elverum are given in Table 1.

Retene has not been detected in air samples from areas not affected by wood combustion analysed in this laboratory or in any other published studies. This indicates that retene may be used as a unique qualitative PAH molecular marker of wood combustion in ambient air. Other polycyclic aromatic compounds which have been proposed as source specific compounds are cyclopenta(cd)pyrene for automobile exhaust²³, benzo(b)-

naphtho(2,1-d)thiophene for hard coal²³ and picene derivatives for brown coal¹³.

Until now, retene had mostly been identified in recent sediments from lakes and oceans^{24–26}, but it has also been reported in levels of pg m^{-3} in aerosols over the rural western USA²¹. The source of retene in these samples has been reported to be from the natural degradation of abietic acid. In sediments this has been confirmed by analysing PAH at various depths and noting that retene does not vary in concentration in the same way as do the other PAH. Several workers have ruled out combustion as a source of retene in environmental samples^{25,26}. The present results demonstrate that this statement is no longer justified, as retene also seems to be a unique molecular marker in air for coniferous wood combustion.

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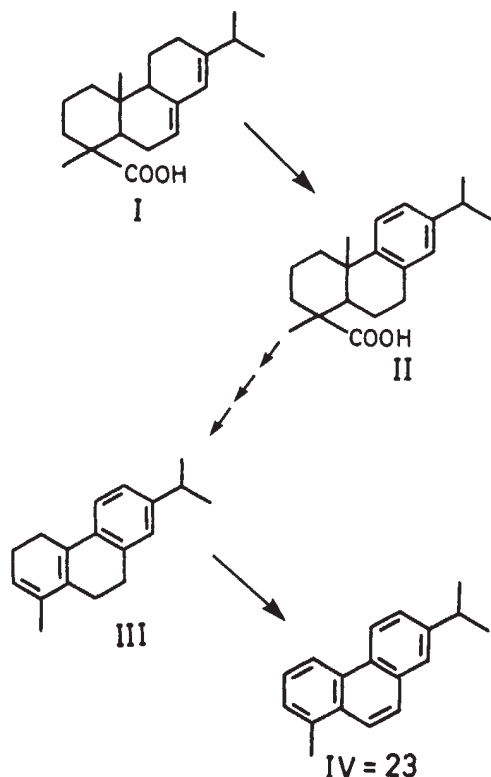


Fig. 2 Hypothetical thermal degradation scheme of abietic acid (I) to retene (IV, 1-methyl-7-isopropylphenanthrene) by coniferous wood combustion.

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Reproductive competition in the communal acorn woodpecker: sisters destroy each other's eggs

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Among group-living vertebrates, highly cooperative or apparently altruistic behaviours usually occur between close relatives^{1,2}. In contrast, highly competitive or selfish behaviours, such as the killing of conspecific young, are often directed towards unrelated individuals^{3,4}. These findings are consistent with predictions of inclusive fitness theory⁵, and have led to the suggestion that kin should receive favoured treatment in the form of not only cooperation but also reduced aggression or competition^{2,6-9}. However, selfish, competitive and manipulative behaviours can evolve among close relatives if the gain in direct (individual) fitness¹⁰ exceeds the loss in indirect fitness^{5,11-14}. We report here an extreme example of reproductive interference between close relatives in the acorn woodpecker, in which communally nesting sibling females in this species destroy their sister's eggs prior to laying their own.

The acorn woodpecker (*Melanerpes formicivorus*) lives in permanently territorial family groups of two to 15 individuals¹⁵. About 20% of groups have two or more breeding females, which are almost always closely related and which cooperate in several forms of communal behaviour, including storing acorns and rearing young¹⁶.

As part of our continuing long-term study at the Hastings Natural History Reservation^{15,16}, we watched 15 nests of 6 different groups containing 2 (in one case 3) breeding females (Table 1). At 12 of the 14 nests with 2 breeding females, the females were known to be either full siblings (four nests) or minimally maternal half siblings and possibly full siblings (eight nests). (It is often not possible to determine exact relatedness because the mating system within groups can be polygynous or polyandrous¹⁷⁻¹⁹). Egg ownership was determined by a combination of continuous observation of the nest hole during the egg-laying period, examination of the nest contents immediately after visits by females, and (in some instances) consistent between-female differences in egg size and shape.

We observed the removal of 52 eggs from the 15 nests. The bird removing one egg was not identified. Of the remaining 51 eggs, 48 (94%) were removed by breeding females and 3 eggs (6%) were removed by breeding males. Of the 48 eggs removed by breeding females (Table 1), egg ownership was determined in 44 cases involving 13 nests. Of these 44 eggs, 35 (80%) were removed by non-maternal breeding females. Nine eggs (20%) were removed by the mother. However, seven of the nine eggs removed by mothers were abnormal 'runt' eggs laid as the first egg of a clutch. Runt eggs lack a normal yolk or are yolkless, never hatch²⁰, and are usually removed. Considering only the 34 normal eggs removed by breeding females, 32 (94%) were removed by non-maternal females and only 2 (6%) were removed by the mother. Non-maternal females that removed eggs were related to the egg in 21 cases, unrelated in 7 cases, and of unknown relationship in 4 cases (Table 1). Thus, the removal of normal eggs from communal nests is performed predominantly by non-maternal breeding females, and the females removing eggs are usually closely related to the eggs they destroy.

Egg destruction in cooperative breeders has been reported previously in groove-billed anis *Crotophaga sulcirostris*²¹ and Mexican jays *Aphelocoma ultramarina*²²; in both species, however, individuals are apparently unrelated or at most only

distantly related to the eggs they destroy. In acorn woodpeckers, a female usually removes eggs from the nest only until she herself lays a normal egg in the nest, presumably because birds are unable to discriminate between their own eggs and those of others. This is similar to what occurs in anis²¹. For 10 acorn woodpecker nests, egg removal ceased when both females laid in the nest simultaneously (five cases) or when the second (or third) female to lay removed some or all of the eggs already present before laying her own first egg (five cases). Thus, egg recognition, such as occurs in ostriches *Struthio camelus*²², is not involved.

Eggs removed from nests are carried intact to a nearby tree and subsequently eaten. Eggs usually were consumed piecemeal by several different individuals, including (in at least four cases) both the female that laid the egg and the sister that removed it. We never observed defence of eggs or aggression among birds eating eggs. These data suggest that nutritional benefits are not the primary advantage accruing to females removing eggs.

As in groove-billed anis²¹, egg removal in the acorn woodpecker results in a communal clutch biased in favour of the bird removing eggs. For all eight communal nests of sibling females where we are confident of egg ownership, the female whose eggs were removed from the nest cavity laid at least as many normal eggs as her sister, but owned a minority of the eggs that were ultimately incubated (Fig. 1).

In spite of this bias, two lines of evidence suggest that lifetime reproductive success for communally nesting females is approximately equivalent. First, for three of four groups where egg destruction was observed among the same sibling pair in two or more years, alternative females destroyed eggs in different years. In another group, both females participated in egg destruction during the same year. Thus, egg destruction is not strongly related to within-group dominance hierarchies, contrary to what has been suggested for anis²¹. Second, in terms of fledged young, the advantage accruing to females removing eggs appears to be relatively small (Fig. 1). Eggs laid late in the laying sequence are more likely to be lost through brood reduction: 20 of 29 (69%) eggs laid on or before the median date in seven successful communal nests produced fledged young compared to only 6 of 22 (27%) eggs laid after the median date ($\chi^2 = 8.70$, d.f. 1, $P < 0.01$). Thus, females that lay second are at a potential disadvantage. By removing early eggs, the second female to lay increases synchrony and minimizes the disadvantages of laying relatively late eggs.

Egg destruction generally does not affect total group reproductive output. Only 63 of 105 normal eggs incubated (60%) in 15 successful communal nests ultimately produced fledged

Table 1 Number of eggs removed by the mothers, non-maternal females and unknown females from 15 communal acorn woodpecker nests.

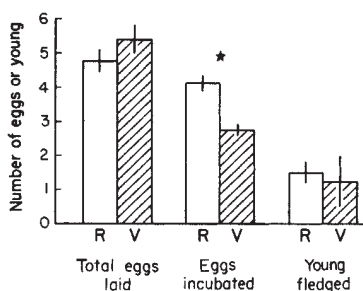
Group	Year	Relatedness of females	Eggs removed by		
			Mother	Non-mother	Unknown
Road 1	1980	Half or full sibs	0	0	1 (0)
	1981	Half or full sibs	1 (1)	5 (0)	0
	1982	Half or full sibs	1 (0)	2 (0)	0
	1983	Half or full sibs	1 (1)	4 (1)	0
Plaque	1980	Full sibs	1 (1)	1 (0)	0
	1981	Full sibs	0	0	0*
	1982	Full sibs	2 (1)	1 (0)	0
Low Hay	1983	Full sibs	1 (1)	1 (0)	1 (0)
	1981	Half or full sibs	0	3 (0)	0
	1982	Half or full sibs	0	1 (0)	0
Dark Hollow	1983	Unrelated	1 (1)	5 (0)	2 (1)
	1982	Half or full sibs	1 (1)	2 (1)	0
Hay Blom Gate†	1983	Half or full sibs	0	4 (1)	0
	1983	Unrelated	0	2 (0)	0
Gate†	1983	Unknown	0	4 (0)	0
	1983	Unknown	0	4 (0)	0
Total			9 (7)	35 (3)	4 (1)

Numbers in parentheses refer to the subset of abnormal 'runt' eggs.

* One egg was removed from this nest by a bird of unknown sex.

† This group contained three breeding females.

Fig. 1 Number of eggs laid, eggs incubated and young fledged (mean $\pm$ s.e.) by the female removing eggs from the nest cavity (R) and the female victim of egg removal (V) in eight nests (four successful nests for young fledged) of communally nesting acorn woodpeckers. The difference in the final number of eggs incubated is significant (Wilcoxon matched-pairs signed rank test $T=0$, two-tailed $P<0.01$).



young. Thus, reproductive success of such groups is limited by factors other than clutch size.

Although acorn woodpecker groups with two communally nesting females have significantly larger clutches¹⁶, on a per female basis they produce fewer offspring than comparable groups with only one breeding female¹⁸. Why, then, does communal nesting occur at all? A direct advantage may accrue to females nesting communally as a result of increased survivorship. Annual survivorship is 70% for females nesting singly compared to 79% for females nesting communally. We estimated the consequences of the contrasting survivorship and reproductive success of communally nesting females on their expected lifetime reproduction by means of a computer simulation. The results of the model suggest that despite egg destruction, communally nesting females can expect to produce at least as many offspring during their lifetime as do singletons: estimated lifetime reproductive success for singleton females is 6.7 fledged young, while for females initially nesting communally estimated lifetime reproductive success is 7.2 offspring. Thus, similar to co-foundresses in *Polistes* wasps^{12,13}, communally nesting females do not appear to suffer a loss in inclusive fitness compared to living on their own, despite egg destruction.

These results confirm the hypothesis that reproductive interference within social groups can be as intense among close relatives as among unrelated individuals^{14,23}. Both cooperation and competitive interference among close kin are compatible with, and indeed predicted by, inclusive fitness theory.

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Neuropeptide Y distribution in human brain

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Tatemoto and Mutt recently used the presence of a C-terminal NH_2 group to identify and isolate a new peptide, neuropeptide Y (NPY), from porcine brain¹. This 36 amino acid peptide was subsequently shown to be active on isolated vas deferens, vascular smooth muscle and pancreatic acinar cells in very low molar concentrations²⁻⁴. In view of these potent effects we have now investigated its distribution in the human brain by radioimmunoassay and immunocytochemistry. High concentrations of NPY have been found, exceeding those of cholecystokinin and somatostatin, hitherto considered to be the most abundant neuropeptides. The distribution of NPY was different from that of any other peptide system described, being particularly concentrated in the basal ganglia, amygdala and nucleus accumbens. Immunocytochemistry demonstrated a large number of NPY neuronal cell bodies especially in the caudate and putamen. Immunoreactive neuronal cell bodies were also clearly localized in cortical areas, particularly layers V and VI. NPY, a newly discovered peptide with potent biological activity, thus seems to be among the most abundant of human neuropeptides. The massive numbers of NPY neurones in the basal ganglia suggest NPY to be of fundamental importance in the control of human motor function.

Human brains were obtained at postmortem from patients without history of neurological or psychiatric disease. The brains were immediately microdissected and then stored at -70°C until extracted in boiling 0.5 M acetic acid (10 ml per g wet weight tissue) for 15 min. Material for immunocytochemistry was obtained postmortem from four subjects (two male, two female, mean age 52 ± 8 yr). Brains were removed whole and perfused via the basilar artery first with 4 l of 0.1 M phosphate-buffered saline (PBS) pH 7.2 and then with 5 l of a 0.4% *p*-benzoquinone solution in PBS. Following perfusion the brains were sliced into 1-cm thick coronal sections and placed in a bath of the same fixative for 8 h (with the slice being turned regularly). Tissue was then rinsed in phosphate buffer containing 30% sucrose before dissection into cryostat blocks. Tissue was processed for immunocytochemistry as previously described⁵.

The distribution of NPY-like immunoreactivity in human brain is shown in Table 1. NPY could be detected in all brain regions investigated. Massive amounts were present in the basal ganglia (caudate and putamen), with relatively large amounts in the limbic system, the amygdala, hippocampus, septal nuclei, hypothalamus and the predominantly motor region of the sensorimotor cortex (Brodmann area 4). Extracts of five regions of human brain (caudate, putamen, hypothalamus, amygdala and nucleus accumbens) were fractionated on a reverse-phase HPLC column as previously described^{6,7}. NPY-like immunoreactivity in human brain extracts eluted in a single peak in a similar, but not identical position to that of the porcine standard (data not shown), suggesting that there may be a minor species difference between human and porcine NPY. The NPY concentrations found in these human brains are closely similar to those obtained from extracts of series of fresh porcine, rat and mouse brain tissue extracted immediately after death (unpublished observations). Avian pancreatic polypeptide

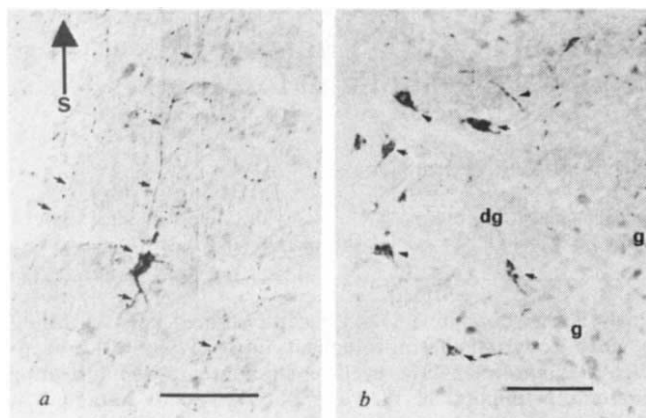


Fig. 1 *a*, NPY-like immunoreactivity in a neuronal cell body, fibres and terminal (arrowed) in the cingulate cortex. *S*, cortical surface. *b*, NPY-like immunoreactivity present in large numbers of neurones (arrowed) in dentate gyrus (*dg*); *g*, granule cell layer. Scale bars, 50 μ m.

Methods: For immunocytochemistry, thin sections of tissue (20 μ m) were cut through the area of interest and then incubated overnight with the specific antiserum to NPY (dilution 1:1,000 in PBS). Sections were then washed and the site of the antibody-antigen reaction was visualized by the peroxidase-antiperoxidase (PAP) method. Preabsorption of the antiserum with NPY (0.1 nmol per ml of diluted antiserum) abolished all specific staining and was used as a control for specificity. Preabsorption of the antiserum with APP, BPP, PYY, somatostatin, VIP, CCK-8, glucagon, ACTH, [Met]enkephalin, substance P, neurotensin or neurophysin (all at 50 nmol per ml of diluted antiserum) had no discernable effect on degree or intensity of immunostaining.

(APP)-like immunoreactivity was undetectable in all human brain regions (<0.5 pmol per g) by radioimmunoassay using a specific and sensitive APP antibody⁸ which had previously been used to stain so-called APP-immunoreactive nerves in mammals⁹⁻¹³.

NPY-like immunostaining neuronal cell bodies were particularly dense in the caudate and putamen. Large numbers of immunoreactive neuronal cell bodies were seen in cortical areas, particularly the deeper layers V and VI (Fig. 1*a*) and subcortical white matter. Moderate numbers of neuronal cell bodies were found scattered throughout the amygdaloid complex and in close association with the pyramidal layer in the hippocampus. A particularly dense concentration of cell bodies was found within the dentate gyrus (Fig. 1*b*). Diencephalic structures contained smaller numbers of scattered cell bodies, with most being found in medial aspects between the septal and preoptic areas. Numerous fibres were found in limbic cortical areas with lower densities in more lateral cortical regions (for example, parietal cortex). These fibres formed dense networks in cortical layers II-IV and appeared to run over the surface of the corpus callosum in layer VI. Immunoreactive fibre networks were less prevalent in hippocampus, amygdala and striatum, while large numbers of immunoreactive fibres were found in midline diencephalic regions and medial brain-stem structures.

The HPLC column elution position and parallel cross-reaction in radioimmunoassay clearly show that human NPY is closely similar to porcine NPY, although proof of its structure must await full purification and amino acid sequence analysis.

NPY shares a substantial sequence homology with APP (20 of 36 residues) and bovine pancreatic polypeptide (BPP, 17 of 36 residues)². Several reports have shown the presence of APP⁹⁻¹³ and BPP¹⁴ in the central nervous system. However, neither peptide has been extracted from brain tissue. Furthermore, using a double-staining elution technique¹⁵ and the same antiserum as other authors⁹⁻¹³, we have found APP-like immunoreactivity and NPY-like immunoreactivity to be co-localized in the same neurones in human (Fig. 2) and rat brain⁷.

Table 1 Distribution of NPY-like immunoreactivity in normal human brain

Human brain area	NPY (pmol per g)
Frontal cortex (BA 4)	120.1 $\pm$ 12.5
Frontal cortex (BA 10)	2.5 $\pm$ 0.3
Temporal cortex (BA 38)	95.5 $\pm$ 33.0
Caudate	534.0 $\pm$ 170.0
Putamen	243.0 $\pm$ 51.0
Globus pallidus (lateral)	43.5 $\pm$ 11.9
Globus pallidus (medial)	27.9 $\pm$ 8.9
Substantia nigra (compact)	32.6 $\pm$ 6.7
Substantia nigra (reticular)	28.9 $\pm$ 3.7
Periaqueductal grey	60.0 $\pm$ 4.1
Thalamus (anteromedial)	20.6 $\pm$ 1.8
Thalamus (anterolateral)	7.1 $\pm$ 0.8
Hypothalamus	182.0 $\pm$ 21.0
Amygdala	262.0 $\pm$ 45.0
Hippocampus	103.0 $\pm$ 20.0
Septal nuclei	85.0 $\pm$ 15.0
Nucleus accumbens	305.0 $\pm$ 78.0
Basal pons	6.6 $\pm$ 1.4
Cerebellar cortex	1.3 $\pm$ 0.2

Results are expressed in pmol per g (mean $\pm$ s.e.m.) wet tissue (three male, two female, mean age 80 yr, range 39-95 yr). NPY concentrations were measured by specific radioimmunoassay using an antiserum (NY7) raised in a rabbit immunized with pure porcine NPY (55 nmol) conjugated with *bis*-diazobenzidine to bovine albumin (15 nmol). Rabbits received a primary immunization of 10 nmol conjugated NPY in a 2 ml Freund's complete adjuvant via four subcutaneous injections into each groin and axilla. Boosts (5 nmol conjugated NPY) were given as above but in incomplete Freund's adjuvant 3 months after the primary and then at monthly intervals thereafter. The third and subsequent boosts of diazo conjugate of NPY used keyhole limpet haemocyanin as carrier. NPY (1 nmol) was iodinated by the conventional chloramine-T oxidation using 0.4 nmol Na¹²⁵I (Amersham IMS 30) and 70 nmol chloramine-T. After mixing for 10 s the reaction was terminated by addition of 200 nmol sodium metabisulphite. Labelled NPY was purified on a 0.9 $\times$ 60 cm Sephadex G-50 superfine column, eluted with 100 mmol l⁻¹ formic acid with 40 μ M BSA at 3 ml h⁻¹. The specific activity of iodinated NPY measured by self displacement was 42 Bq fmol⁻¹. The assay was set up in 0.8 ml 50 mmol l⁻¹ phosphate buffer pH 7.4 containing 12 μ mol l⁻¹ BSA, 2 fmol labelled NPY, antiserum at a final dilution of 1:24,000 and a maximum of 10 μ l tissue extract. After incubation at 4 $^{\circ}$ C for 5 days the free peptide was separated from bound by addition of 4 mg charcoal (Norit GSX, Hopkin & Williams) with 400 μ g dextran (70T, Sigma) in 0.5 ml assay buffer. After centrifugation at 2,000g for 20 min at 4 $^{\circ}$ C the supernatant was separated from the carbon pellet and both the free and bound fractions were counted. The assay is capable of detecting changes between adjacent assay tubes of 2 fmol with 95% confidence and showed no cross-reaction with porcine PP, porcine peptide YY (PYY) or APP in concentrations up to 100 pmol per tube. APP-like immunoreactivity was measured using the method previously described in detail⁶. This antiserum, used at a dilution of 1:160,000 (Kimmel 622), with 2 fmol per tube iodinated APP, could detect changes between adjacent tubes of 0.8 fmol per tube with 95% confidence. This assay showed no cross-reaction with porcine PP, porcine PYY or porcine NPY in concentrations up to 100 pmol/tube.

We interpret the HPLC data and these immunocytochemical findings as strong evidence that NPY is the endogenous PP-peptide in the mammalian brain and that earlier reports on the distribution of and co-localization with other transmitter candidates of APP- and BPP-like peptides in the central nervous system⁹⁻¹⁴ were a result of antibody cross-reactivity due to the large sequence homologies between the peptides.

The detailed distribution of NPY in the human brain differs from that of any other peptide so far mapped. For example, cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP), among the most abundant peptides, were found in these brains in highest concentration in the cortex (CCK 100-200 pmol per g and VIP 15-30 pmol per g), lower amounts being found in most other regions including the basal ganglia. In addition, concentrations of NPY in the hypothalamus exceed

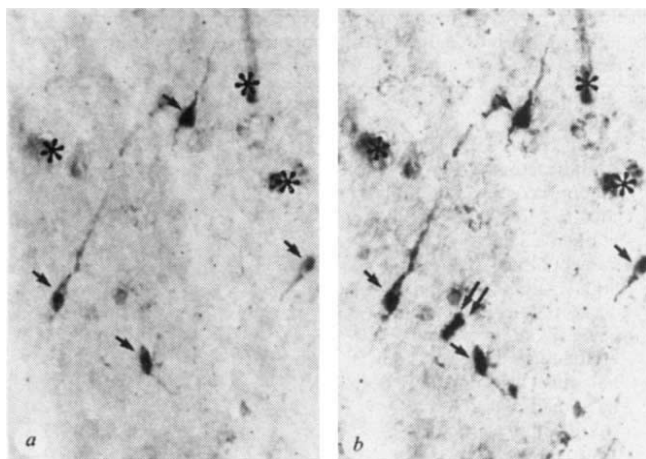


Fig. 2 The double staining procedure shows APP-like immunoreactivity (a) and NPY-like immunoreactivity (b) to be present in the same neurones in the cortex (arrowed). Using this method all structures positive for APP were also found to contain NPY. * Blood vessels, nonspecific deposit. $\times 250$.

Methods: Sections were immunostained by the PAP method as for Fig. 1 with APP antiserum (dilution 1:2,000) and developed in a 0.5% solution of 4 chloro-1-naphthol in PBS and 0.2% H_2O_2 . After photographing, the blue reaction product was removed by rinsing in alcohol and the immunocomplex eluted in 0.1 M glycine-HCl buffer¹³. Control sections were removed at intervals and developed to check for total elution. Sections were then reincubated with NPY antiserum, developed in diaminobenzidine and the brown reaction product was re-photographed.

even those of somatostatin (160–200 pmol per g). The concentrations of these and other neuropeptides in the brains fell well within the ranges reported by several other groups for human brain^{16–20}. NPY differed in its regional distribution within the cortex, moderate levels being found in Brodmann areas 38 and 4 (limbic and sensorimotor cortex, respectively) and relatively low levels in area 10 (medial and lateral aspects of the frontal pole). This may be relevant to the high NPY-like immunoreactivity in both limbic regions (amygdala and hippocampus) and striatal areas (caudate nucleus and putamen).

The newly isolated and biologically potent peptide NPY is thus the single most abundant neuropeptide yet found and has a distinctive pattern of distribution. It must be considered an important putative neurotransmitter or neuromodulator in the human brain and, in view of the massive striatal concentrations, must be of particular interest in the extrapyramidal motor system.

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Dopaminergic D-3 binding sites are not presynaptic autoreceptors

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Postsynaptic dopamine (DA) receptors have been classified biochemically and pharmacologically into two types: D-1 receptors mediate adenylate cyclase stimulation, demonstrating micromolar affinity for DA and butyrophenone antagonists; D-2 receptors mediate adenylate cyclase inhibition, demonstrating nanomolar affinity for DA and butyrophenone antagonists^{1,2}. D-1 receptors are labelled by 3H -thioxanthene antagonists^{2,3}, while D-2 receptors are labelled by both 3H -agonists and all 3H -antagonists^{2,4}. A third class of dopaminergic binding site, termed D-3, represents high-affinity 3H -agonist binding sites demonstrating low, micromolar, affinity for butyrophenones^{5,6}. In the rat striatum, D-3 sites were decreased 50% by 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal DA pathway^{7–9}, suggesting that such D-3 binding labels presynaptic DA autoreceptors on nigrostriatal terminals^{5,7–9}. However, nigrostriatal denervation produces a concomitant depletion of striatal DA^{10,11}. Here we demonstrate that a reserpine-induced depletion of DA produces a decrease in D-3 binding comparable to that seen with nigrostriatal denervation, independent of presynaptic terminal degeneration. This loss in binding, or that caused by 6-OHDA lesions, is recovered by preincubating the striatal membranes with DA or with the supernatant from control striatal membrane preparations. We therefore suggest that the loss of D-3 binding following 6-OHDA lesions results from the depletion of endogenous DA rather than the degeneration of terminals and their putatively associated autoreceptors.

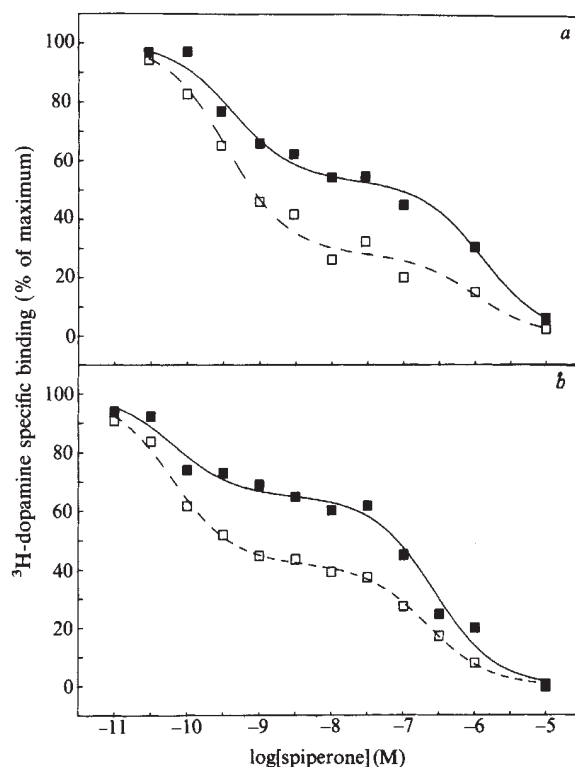
It has been shown previously that 3H -agonists can label both the classical postsynaptic D-2 receptors and D-3 binding sites⁴. Figure 1 shows that butyrophenone 3H -DA competition curves are biphasic; that is, 3H -DA may bind to two independent sites, one having high (nanomolar) and one having low (micromolar) affinity for butyrophenone antagonists. Since unlabelled butyrophenones such as spiperone compete with a portion of 3H -agonist binding with affinities comparable to 3H -butyrophenone affinities for D-2 DA receptor binding sites (Fig. 1 and refs 2, 4, 5), spiperone is useful in distinguishing between 3H -agonist binding to D-2 and D-3 DA binding sites. In defining D-2 and D-3 binding by their respective high and low affinity for butyrophenones, weighted nonlinear regression computer analyses^{12,13} of these competition curves to quantify binding parameters demonstrate that about 40–50% of 3H -DA binding is to D-2 receptors and 50–60% to putative D-3 binding sites.

Six to eight weeks following a 6-OHDA lesion of the nigrostriatal tract (Fig. 1a, dashed lines) the proportion of D-2 3H -DA binding is unchanged or slightly increased, with a concomitant decrease of $43 \pm 6\%$ of D-3 3H -DA binding site density. The total amount of 3H -DA-specific binding decreases under this protocol by $27 \pm 4\%$. Figure 1b demonstrates that similar changes in displacement curves are obtained following a 20-h reserpine-induced catecholamine depletion of normal rats which produces a $42 \pm 6\%$ decrease in D-3 binding. This decrease in D-3-specific binding is not the result of a direct reserpine interaction with D-3 receptors, since *in vitro* reserpine is a poor inhibitor of D-3 binding ($IC_{50} \gg 10 \mu M$).

In separate experiments, saturation analyses of D-3-specific 3H -DA binding in 6-OHDA lesioned versus contralateral striata were conducted in the presence of a concentration of spiperone (10 nM) which inhibited 3H -DA binding to D-2 binding sites at all 3H -DA concentrations used (0.2–15 nM). The combined

Fig. 1 Effect of 6-OHDA lesions (a) and acute reserpine administration (b) on spiperone competition for ^3H -DA binding sites in rat striatum. **a**, Unilateral 6-OHDA lesions. Representative fitted curves for spiperone/ ^3H -DA competition in contralateral (■) and lesioned (□) striata. Tissue preparation and ^3H -DA binding were conducted as described previously⁴ (see Table 1 legend). Representative spiperone competition for ^3H -DA binding experiments in control striatum were analysed showing a best-fit curve modelling to two sites. Analyses were conducted constraining ^3H -DA dissociation constants at each site to values pre-determined in saturation experiments on control and experimental tissue. K_D values for binding to D-3 sites were constrained to: contralateral = 6 nM; 6-OHDA lesioned = 10 nM. Final parameter estimates shown below assume that the affinity of ^3H -DA for D-2 sites is identical in lesioned and control striata. K_D values for D-2-selective ^3H -DA binding were constrained to 6 nM for control tissue and 6 nM or 10 nM for lesioned tissue as suggested by saturation of total specific ^3H -DA binding. However, constraining K_D (D-2) values in analyses of lesioned tissue to either value (6 or 10 nM) did not significantly alter results found for D-3 binding parameters. In the control curve shown, K_i values for spiperone for the two sites were estimated to be 340 pM and 1.06 μM for the high-affinity (D-2) and low-affinity (D-3) sites, respectively. D-3 sites comprised 54% of the total at this ^3H -DA concentration (1.3 nM) in the non-lesioned striatum. On the lesioned side D-3 sites comprised only 36.5% of the total ^3H -DA binding, or 68% of the concentration of D-3 sites observed in the contralateral side. Mean values $\pm$ s.e.m. for final parameter estimates ($n=6$) (K = dissociation constant; R = receptor concentration): Contralateral: $K_{D-2} = 220 \pm 37$ pM, $R_{D-2} = 29.5 \pm 1.2$ fmol per mg tissue; $K_{D-3} = 2.32 \pm 0.62$ μM , $R_{D-3} = 34.8 \pm 1.9$ fmol per mg; Lesion: $K_{D-2} = 162 \pm 28$ nM, $R_{D-2} = 33.7 \pm 2.6$ fmol per mg tissue; $K_{D-3} = 1.25 \pm 0.40$ μM , $R_{D-3} = 19.4 \pm 1.8$ fmol per mg tissue ($*P < 0.005$ (paired t -test) compared with contralateral R_{D-3}). ^3H -DA saturation experiments indicated that the decrease in D-3 binding reflected a decrease in B_{max} and an increase in K_D . Qualitatively identical results were found for the agonist ^3H -apomorphine. For control striata total ^3H -DA binding c.p.m. were 800 and 300 per filter for total and blank, respectively. **b**, Fitted curves for spiperone/ ^3H -DA competition in the striatum of saline (■) and reserpine (□) injected rats. Similar changes in the D-3 components of ^3H -DA binding were seen in reserpine-injected rats as for 6-OHDA-denervated striatum. However, decreases in D-3-specific ^3H -DA binding are due to a decrease in B_{max} only. The K_D s of ^3H -DA for both sites in both treated and untreated animals were constrained to 5 nM. Mean values $\pm$ s.e.m. ($n=6$) were: Control: $K_{D-2} = 143 \pm 60$ pM, $R_{D-2} = 7.00 \pm 0.69$ fmol per mg tissue; $K_{D-3} = 216 \pm 25$ nM, $R_{D-3} = 12.06 \pm 0.69$ fmol per mg tissue; Reserpine: $K_{D-2} = 153 \pm 53$ pM, $R_{D-2} = 7.50 \pm 1.12$ fmol per mg tissue; $K_{D-3} = 227 \pm 38$ nM, $R_{D-3} = 6.81 \pm 0.56$ fmol per mg tissue [$*P < 0.005$ (paired t -test) compared with control R_{D-3}]. Experiments for **a** and **b** were conducted at different times which contributed to the differences seen in the absolute binding parameters of the control striata.

Methods: Unilateral 6-OHDA lesions were made in male Sprague-Dawley rats (200–250 g) placed in a David Kopf stereotaxic apparatus under nembutal anaesthesia. 6-OHDA (8 μg in 4 μl of saline, 0.1% ascorbic acid; 5 min infusion) was injected into the substantia nigra at coordinates $A = 3.2$ mm; $L = 1.4$ mm; $V = -7.6$ mm, and the animals were allowed 6–8 weeks' survival. Extent of lesion was monitored by striatal tyrosine hydroxylase activity^{19,20}. Only data from rats showing $>95\%$ losses in tyrosine hydroxylase activity (compared with activity in the contralateral striatum) were included in the analysis. Reserpine (Serpasil; Ciba), 4 mg per kg s.c., was injected and the rats were killed 20 h post-injection. Curves were analysed using LIGAND, a weighted nonlinear least-squares analysis program as described in detail previously^{12,13} using the UCSD VAXII. Ordinate values represent per cent maximum specific binding in each tissue.



data from two independent studies (six experiments) indicate that the decrease in D-3 binding after 6-OHDA-induced nigrostriatal denervation is due to both a 20% decrease in B_{max} and a 55% increase in K_D of ^3H -DA for these sites. In reserpine-treated animals, however, the decrease in D-3 binding is due entirely to a decrease in B_{max} (35%, $n=7$). Importantly, preincubating striatal membranes from 6-OHDA-denervated striata with DA reverses the 'lesion-induced' loss in D-3 ^3H -DA binding (Table 1)*, and both ^3H -DA affinity and B_{max} values for D-3 binding sites return to normal with this DA preincubation.

Furthermore, preincubating membranes from reserpine-treated animals with either DA (100 nM) or the supernatant from a membrane preparation of a normal striatum also reverses the loss in 'D-3' specific ^3H -DA binding (Table 1). The effects of reserpine and 6-OHDA lesion on D-3-specific ^3H -DA binding are not additive (Table 1), and preincubating membranes with the dopaminergic agonist ($\pm$)2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene also reverses the reserpine-induced loss in D-3-specific ^3H -DA binding. However, preincubations with catechol or the β -adrenergic agonist isoprenaline (100 nM) do not reverse reserpine-induced losses in D-3 binding. Thus, the loss in striatal D-3 binding produced both by reserpine treatment and by 6-OHDA denervation appears to result from a depletion of DA in the treated tissues. (^3H -DA binding is divided into D-2 and D-3

components by incubating in the presence of 30 nM spiperone. As shown in Fig. 1, this concentration of spiperone falls on a plateau in the curve and thus blocks ^3H -DA binding to the site having high affinity for spiperone (D-2). The site having low affinity for spiperone is unaffected. Thus, D-2-specific ^3H -DA binding is defined as the difference in ^3H -DA bound in the presence and absence of 30 nM spiperone. D-3-specific binding is defined as the specific ^3H -DA binding remaining in the presence of 30 nM spiperone. *cis*-Flupentixol (10 μM), a dopamine antagonist at both D-2 and D-3 binding sites, is used as a blank for D-3 binding sites⁴.)

We see, as others have also reported^{4,14}, that DA added during the preincubations of washed or DA-depleted striatal membrane homogenates produces increased D-3 binding. In these studies the preincubation of the membrane homogenates was a crucial variable in the improved expression of all ^3H -DA-specific binding. As has been suggested by Hamblin and Creese⁴, this preincubation in the presence of (endogenous) DA and divalent cations may act to promote the dissociation and washout of endogenous guanine nucleotides from guanine nucleotide binding sites which regulate DA agonist binding to D-2 and D-3 binding sites. This resembles the mechanism by which agonist preincubations seem to enhance agonist binding to some β -adrenergic receptors¹⁵. Irrespective of mechanism, our data indicate that 6-OHDA denervation and reserpine treatments

Table 1 Dopamine reversibility of 6-OHDA denervation and reserpine-induced declines in D-3-specific ^3H -dopamine binding in rat striatum

			(n)	^3H -Dopamine binding (fmol per mg tissue)	
				D-2	D-3
Expt 1	Striatum Contralateral Lesion	Preincubation Vehicle	(7)	1.06 $\pm$ 0.17 _{NS}	2.19 $\pm$ 0.23*
				0.83 $\pm$ 0.12 _{NS}	0.71 $\pm$ 0.14
	Contralateral Lesion	+ 100 nM DA	(5)	1.46 $\pm$ 0.18 _{NS}	3.40 $\pm$ 0.14 _{NS}
				1.91 $\pm$ 0.19 _{NS}	3.46 $\pm$ 0.11 _{NS}
Expt 2	Striatum Contralateral Lesion	Treatment Reserpine	(6)	0.66 $\pm$ 0.11 _{NS}	0.91 $\pm$ 0.14 _{NS}
				0.79 $\pm$ 0.19 _{NS}	0.78 $\pm$ 0.20 _{NS}
Expt 3	Treatment Saline	Preincubation Vehicle	(6)	0.87 $\pm$ 0.18 _{NS}	2.19 $\pm$ 0.21 _{NS}
		+ 100 nM DA		0.79 $\pm$ 0.10 _{NS}	2.53 $\pm$ 0.21 _{NS}
	Reserpine	Vehicle	(6)	0.60 $\pm$ 0.03 _{NS}	0.96 $\pm$ 0.10 _‡
		+ 100 nM DA		0.84 $\pm$ 0.22 _{NS}	2.16 $\pm$ 0.13*
Expt 4	Treatment Saline	Supernatant Saline	(6)	0.40 $\pm$ 0.07 _{NS}	2.36 $\pm$ 0.19*
		Reserpine		0.36 $\pm$ 0.08 _{NS}	1.53 $\pm$ 0.22
	Reserpine	Reserpine	(6)	0.47 $\pm$ 0.08 _{NS}	0.78 $\pm$ 0.09 _‡
		Saline		0.42 $\pm$ 0.05 _{NS}	2.02 $\pm$ 0.25 _‡

Expt 1: 6-OHDA denervation; the effects of preincubating in the presence of DA. To the preincubation homogenates suspended in Tris-HCl/MgSO₄ were added either DA (100 nM final concentration in dilute HCl) or dilute HCl vehicle. Homogenates were incubated for 15 min at 37 °C as described below. The addition of DA to the preincubation reverses the loss in D-3 binding seen on the lesioned side. Similar results were obtained when ^3H -apomorphine was used as the labelled ligand. Expts 2–4: reserpine (4 mg per kg) was injected 20 h before death. In expt 2, reserpine's effects on D-3 binding were not additive with the effect of 6-OHDA denervation. Expt 3 shows that the addition of DA to the preincubation of homogenates from reserpine-treated rats reverses the observed loss of D-3 binding. Expt 4: acute reserpine supernatant exchange. **Methods:** Binding assays for ^3H -DA were conducted as described in detail elsewhere⁴. Briefly, rat striata were homogenized in 100 volumes of ice-cold Tris-HCl pH 7.7 (25 °C). Homogenates were centrifuged for 10 min at 50,000g and the supernatant was discarded (except for supernatant exchange experiments). These pellets were resuspended in 100 volumes Tris-HCl containing 2 mM MgSO₄ and incubated for 15 min at 37 °C. To terminate this incubation, an equal volume of ice-cold Tris-HCl was added before centrifuging the samples at 50,000g for 10 min. The pellet was washed once more in Tris-HCl before resuspending the final pellet in assay buffer containing 20 mM MOPS, 1 mM EDTA-free acid, 0.1% ascorbic acid, 4 mM MgSO₄, 10 μM pargyline, and Tris base to yield a final assay pH of 7.2 at 22 °C. Incubation volumes were 1 ml, and ^3H -DA was 1.2–1.5 nM (final concentration; 40.0–48.0 Ci mmol⁻¹, NEN). Incubations (22 °C) were terminated after 90 min by vacuum filtration of the tissue through Whatman GF/C filters followed by standard scintillation spectroscopy. Preliminary experiments determined: that this assay pH and filter wash were optimal; that the inclusion of EDTA in the assay improved receptor stability but had no direct effect on binding; that the inclusion of ascorbic acid did not significantly affect specific binding but decreased nonspecific binding; and that the tissue concentrations used were within the range of tissue linearity. Nonspecific binding was determined in the presence of 30 nM spiperone for D-2-specific sites and in the presence of 10 μM *cis*-flupentixol for D-3-specific sites. Total binding for D-3-specific sites was determined in the presence of 30 nM spiperone. All values were determined in triplicate with animal replicates listed in parentheses. For expt 4, the first homogenization of both (pooled) striata from each animal was conducted in Tris-HCl/MgSO₄ buffer. The tissue homogenate was split into two tubes and centrifuged as described previously. Samples from reserpine- and saline-injected animals were paired and samples were resuspended in either their own supernatant or that of their paired opposite. Preincubations, tissue preparations and binding assays proceeded as described above. Incubating the membranes from reserpine-treated rats with the first supernatant from control rats reverses the observed loss in D-3 binding normally produced by reserpine.

* $P < 0.001$, † $P < 0.005$, for paired *t*-tests (two-tailed); ‡ $P < 0.001$, compared with saline-injected animals. NS, not significant.

appear to reduce specific D-3 binding by eliminating the usual preincubation-induced enhancement of this binding seen in control tissues containing endogenous DA. However, we cannot explain why chronic dopaminergic denervation of the striatum should affect changes in both K_D and B_{max} of ^3H -DA binding to D-3 sites while acute reserpine treatments affect only B_{max} values.

Previously, Schwartz and colleagues have demonstrated similar changes in D-2 and D-3 components of ^3H -agonist binding following 6-OHDA lesion^{8,9}. They concluded that the lost D-3 sites were presynaptic. However, the important DA depletion controls were not undertaken. The present results suggest that D-3 binding sites are not presynaptic but are postsynaptic, both because reserpine-induced dopamine depletion also produces decreased ^3H -agonist binding to D-3 sites, and because no loss in binding is seen when DA is added to tissue preparations. This conclusion is reinforced by our preliminary findings that kainate lesion of the striatum, which destroys neuronal cell bodies, reduces both D-2 and D-3 ^3H -DA binding by more than 70%.

In β -adrenergic systems associated with the stimulation of cyclic AMP production there is a guanine nucleotide-sensitive high-affinity agonist binding state of the receptor in membrane preparation^{16,17}. Recent studies have indeed suggested that D-1 DA receptors demonstrate high- and low-affinity agonist binding states which are modulated by guanine nucleotides¹⁸. We suggest, therefore, that D-3 binding may, at least in part, represent a high-affinity agonist binding state of the D-1 receptor.

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Note added in proof: Since original submission of our findings, similar results have been reported by Schweitzer and Bacopoulos (*Life Sci.* **32**, 531–540; 1983).

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A novel development pattern for frogs: gastrulation produces an embryonic disk

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According to von Baer's laws of 1828^{1,2}, early embryos of closely related animals tend to resemble each other. While the application of these laws to a large taxonomic group, such as the vertebrates, has been strongly criticized³, early development appears similar within classes of terrestrial vertebrates. One reason for the similarities may be that embryos of a given taxonomic group are subjected to similar environmental conditions. It follows that similar animals which provide their embryos with different environments may have different developmental patterns. Amphibians present several modes of reproduction^{4,5} and are therefore particularly suited to the examination of this issue. While many amphibians have small eggs and aquatic development, the egg-brooding hylid frogs exhibit maternal incubation of embryos, multinucleate oogenesis^{6,7}, and very large eggs^{5,8}. We have compared early development of the egg-brooding hylids, *Gastrotheca riobambae* and *Gastrotheca plumbea*, with *Eleutherodactylus coqui* and *Xenopus laevis*. We report here that early developmental pattern in *Gastrotheca* differs from other frogs, particularly with respect to gastrulation, which results in the formation of an embryonic disk.

To observe development, we removed embryos of *G. riobambae* from the pouch on the mother's back and when necessary, maintained them for several days in a humid chamber or with a small drop of modified Barth's solution⁹ under mineral oil. Embryos were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, in Smith's solution¹⁰ or in Stocker's fixative followed by silver nitrate staining¹¹. The latter procedure stains cell borders making the size and shape of each surface cell

visible, an important advantage since the embryos themselves lack pigment and are uniformly pale.

Despite the large size of the egg (3 mm in diameter), the cleavage divisions completely divide it, and the pattern of cell movements during gastrulation is basically similar to other frogs. As gastrulation is completed, however, a novel structure forms around the closing blastopore, and we call this structure the embryonic disk (Fig. 1a).

The disk forms from the cells of the fusing blastoporal lips (Fig. 1b), and it consists of a distinct group of smaller cells (Fig. 2a, b). The disk usually has a raised border and is separated from the yolk cells by the archenteron, the primitive gut (Fig. 2c). The diameter of the disk is about half that of the embryo, and therefore, the disk represents about 7% of the surface area. It is originally about 10–15 cells thick. Outside the disk, the embryo consists of large yolk cells covered by a thin epithelium which is one cell thick (Fig. 2d).

The embryonic disk remains the same size for 2 days after which time it undergoes a marked expansion concomitant with the enlargement of the archenteron (compare Fig. 2c, e). The expansion of the disk is primarily due to stretching rather than to recruitment of bordering cells, as determined by marking the embryo with spots of Nile blue sulphate, a vital stain. Spots placed outside the disk remained outside, and some marks placed in the disk became stretched, particularly those placed in the future anterior regions of the body (Fig. 3). Due to the anterior expansion of the disk, the blastopore, which had been more or less central in the disk, ends up at the posterior end of the developing body axis.

When we follow the subsequent development of the embryo (Fig. 1c), we find that almost all of the animal's body comes from what was originally the small embryonic disk. The embryo looks bird- or reptile-like as it initially develops as several sheets of tissue which secondarily form tubes by body folds⁸. The embryonic disk of *G. riobambae*, however, is not homologous with those of other vertebrates. In *G. riobambae*, the disk forms as a result of the cell movements of gastrulation, while in caecilians¹², reptiles, birds, mammals and some fish, a small disk of cells is present prior to cell movements.

To see whether an embryonic disk is found in the development of other frogs, we examined gastrulae of *G. plumbea*, *E. coqui* and *X. laevis*. *G. plumbea*, whose egg is about 4 mm in diameter, appears to develop like *G. riobambae* with the formation of a disk. *E. coqui* has a large (3.3 mm) white egg which is laid on land and brooded by the male. Despite these reproductive adaptations, cleavage and gastrulation are similar to frogs with aquatic development, and there is no embryonic disk. Finally, *X. laevis*, the most popular frog for laboratory research, has a small (1.4 mm) egg which is laid in water. Neither silver-staining of embryos from an albino female nor sections gave an indication of a disk.

When we compare late gastrulae of *G. riobambae* and *X. laevis* (Fig. 1b, e), there are two major differences which account for the presence of a disk in the former but not in the latter. First, in the *G. riobambae* gastrula, the small yolk-poor cells are accumulated around the closing blastopore while in the *X. laevis* gastrula, those smaller yolk-poor cells are spread all the way around. Second, in *X. laevis*, the closing of the blastopore and the anterior extension of the archenteron occur at the same time, while in *G. riobambae*, these two events are temporally separated. This temporal separation may be due to the extremely slow rate of development in *G. riobambae*. Its embryo requires about 1 week for the cleavage divisions and another week for gastrulation, while in *X. laevis*, gastrulation is completed within 1 day of fertilization. The slow rate of development in *G. riobambae* is not simply due to the yolk content, since the large eggs of *E. coqui* complete gastrulation about 3 days after fertilization.

Thus the embryos of *Gastrotheca* illustrate that it is possible to modify greatly the pattern of early development without altering the basic adult morphology. The modification of the pattern of development is probably due to the reproductive

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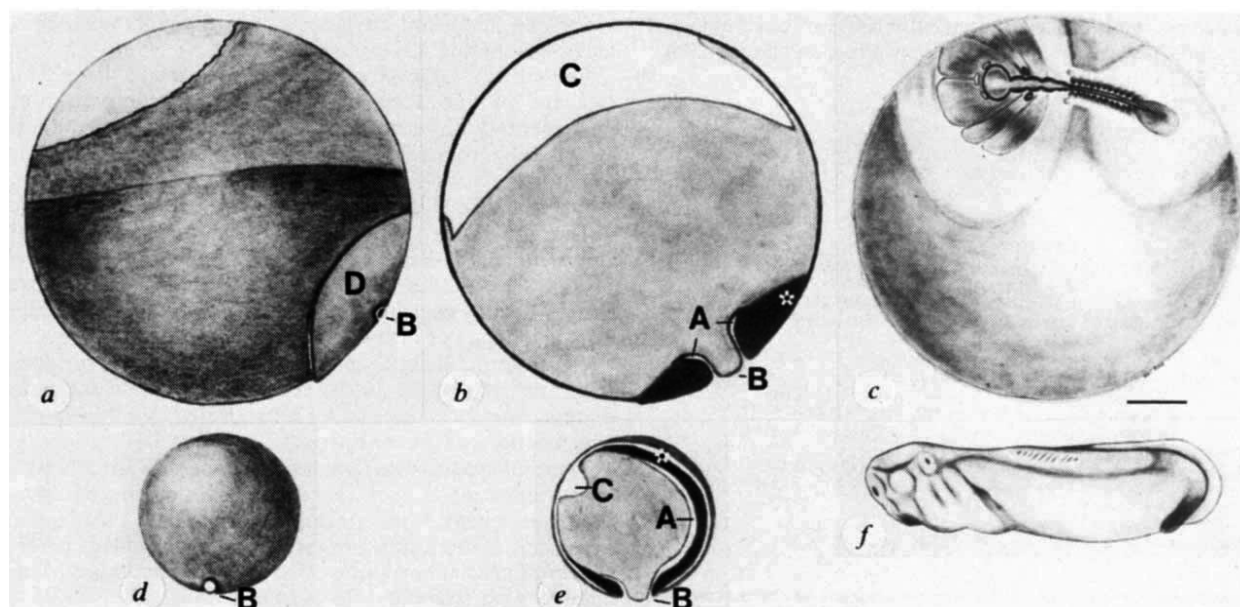


Fig. 1 Comparison of embryos of *Gastrotheca riobambae* and *Xenopus laevis*. *a-c* Show *G. riobambae* embryos and *d-f* show *X. laevis* embryos at comparable stages. *a*, At the end of gastrulation in *G. riobambae*, the blastopore (B) closes, and the embryonic disk (D) forms. This figure represents a stage 8 embryo and has been modified from the normal table in ref. 8 to include the embryonic disk. *b*, A mid-sagittal section of a slightly younger embryo illustrates the large blastocoel cavity (C), the small archenteron (A), and the accumulation of yolk-poor cells (coloured black) around the closing blastopore. The asterisk indicates the area which will form the head of the embryo. *c*, As the disk expands to form the archenteric roof, the whole embryo rotates so that the roof is uppermost. Most of the embryo's body then develops from the expanded disk. *d*, The late gastrula (stage 12½) of *X. laevis* does not have an embryonic disk. *e*, At the time of blastopore (B) closure in *X. laevis*, the extension of the archenteron (A) anteriorly and the distribution of yolk-poor cells (coloured black and darker grey) are very different from the situation in *G. riobambae* (redrawn from ref. 13). *f*, The *X. laevis* embryo (redrawn from ref. 14) has been matched with the *G. riobambae* embryo (*c*) with respect to development of gill arches, eye, and otocyst. The *G. riobambae* embryo is spread on the yolk while the *X. laevis* embryo is elongated showing its adaptation for early swimming. (Scale bar, 0.5 mm.)

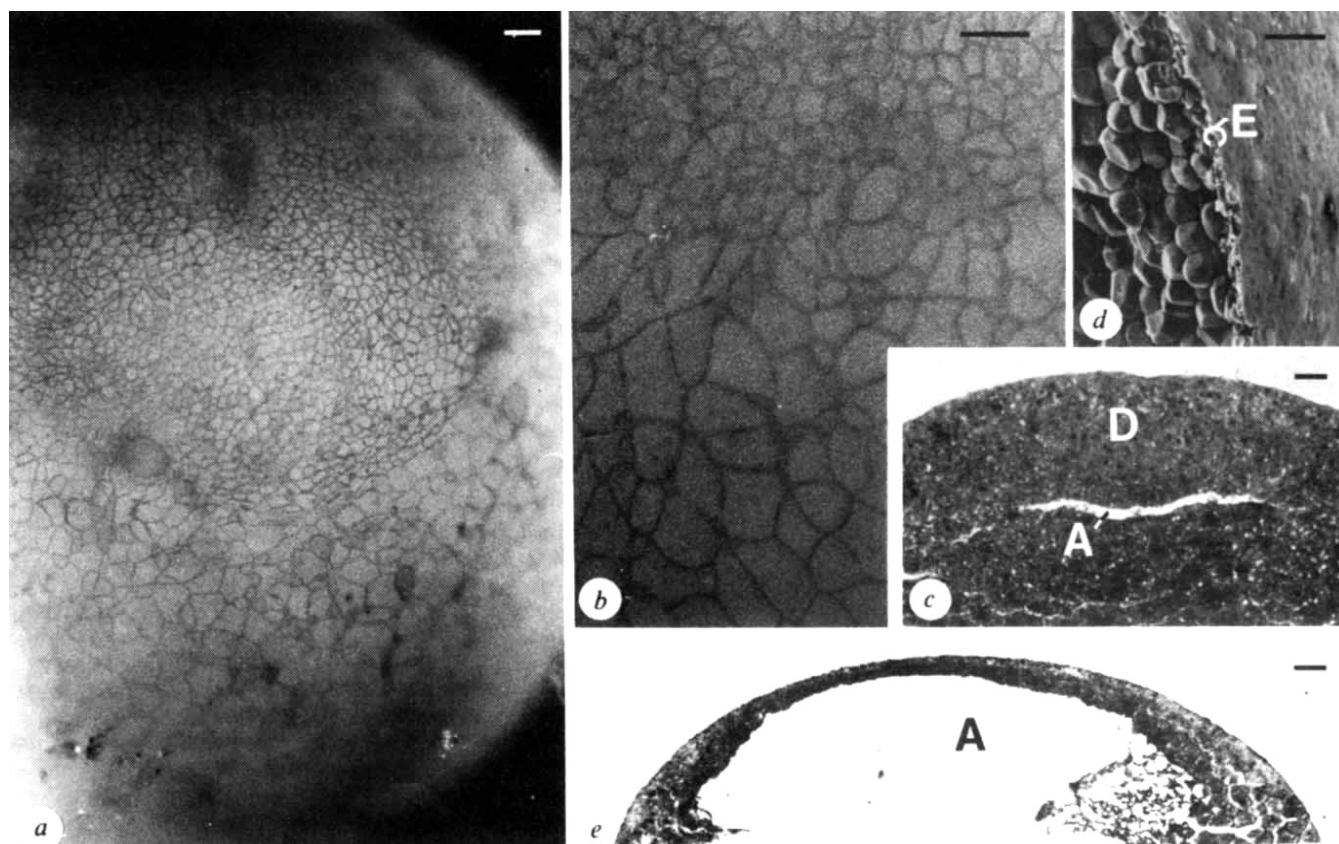
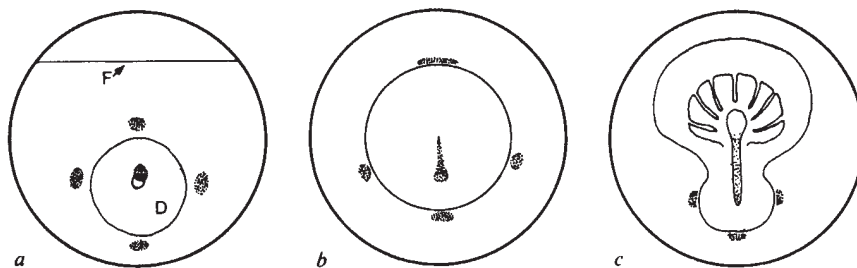


Fig. 2 The embryonic disk. *a*, When embryos at stage 8 are silver-stained, the embryonic disk appears as a group of smaller cells. *b*, There is a clear boundary between the cells of the disk and the rest of the embryo. *c*, As seen in section, a slit-like archenteron (A) separates the small cells of the disk (D) from the large yolk cells. *d*, Outside the area of the disk, the large yolk cells are covered by a thin epithelium (E) which is one cell thick. *e*, With further development, the archenteron expands, and the disk is stretched to form the roof of the archenteron. (Scale bar, 0.1 mm.)

Fig. 3 Vital staining of the embryonic disk. *a*, In a typical experiment, vital stain marks were placed outside the disk (D) and near its centre. The floor of the blastocoel (F) can be seen due to the lesser opaqueness of the area above the floor. This is the area where cells are moving from the floor along the roof (Fig. 1*a*, *b*). *b*, As the disk expands, the outside marks tend to stay outside the disk, and marks within the disk are stretched. *c*, The anterior end of the embryo usually arises from the area of the disk closest to the floor of the blastocoel.



adaptations in these frogs which provide a different set of environmental conditions for the embryo.

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Differential expression of *bithorax* complex genes in the absence of the *extra sex combs* and *trithorax* genes

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Each body segment of *Drosophila* follows a unique developmental pathway, controlled by the selective expression of homeotic genes such as *Sex combs reduced* (*Scr*)^{1,2} and the *bithorax* complex (*BX-C*)^{3,4}. Little is known about the regulation of these genes, though several potential activators or repressors have been described^{5–8}. For instance, absence of the *extra sex combs* (*esc*) gene product apparently causes adventitious expression of all the *BX-C* genes in most or all larval body segments⁵. Absence of the *trithorax* (*trx*) gene appears to prevent *Scr* and *BX-C* expression^{7,9,10} but only in adult cells; differentiation of the larval segments is only slightly affected¹¹. I show here that the correct segmental differentiation of the larva does not require maternally deposited *trx*⁺ product, but that the *esc* mutant phenotype is suppressed by the removal of the *trx* gene, which implies that the *BX-C* can be differentially expressed in the absence of both the *trx* gene and the *esc* gene product.

The *trx* gene is required for the correct differentiation of most regions of the adult epidermis. [The *trx* alleles have been shown to be allelic to the *Rg-bx* mutation¹⁰. I propose that the locus be named *trx* since this name and symbol conforms to the convention for *Drosophila* gene nomenclature.] Hypomorphic *trx* alleles cause transformations of thoracic and abdominal segments towards the mesothoracic and first abdominal segments respectively⁷. Complete removal of the *trx* gene during the larval stages results in the transformation of adult head, thoracic and genital structures towards mesothorax (refs 9, 10 and in preparation). Together these results imply that the *trx* gene is necessary for the expression of at least the *Scr* and *BX-C* genes.

Paradoxically, absence of the *trx* gene has no equivalent effect on larval differentiation. In larvae homozygous for null alleles or deficiencies of the *trx* gene (*trx*[−]), the head and prothoracic segments differentiate normally, although the metathoracic (T3) segment shows some evidence of transformation to mesothorax

(T2) (Fig. 1). In the more posterior abdominal segments (A6–A8) modifications of their ventral cuticular pattern suggest transformation to a more anterior abdominal segment¹¹. These transformations vary between individuals, but there is no evidence of abdominal segments being transformed towards the thoracic level or even to segment A1. Thus the selective expression of the *Scr* and *BX-C* genes is not prohibited when the *trx* gene is absent, though expression of the *BX-C* genes is sub-optimal in the more posterior segments. This lack of a major effect on larval development could be due to maternally contributed *trx*⁺ product⁶ in the egg. To test this, females which produce eggs from germ cells lacking the *trx*⁺ gene were generated. Since *trx*[−] animals do not survive to adulthood, clones of *trx*[−] germ cells were induced by mitotic recombination in flies carrying a *trx*⁺ allele (Fig. 2). Females bearing such clones were mated to males genotypically *trx*[−]/+. Ten clones were identified as being *trx*[−] (Table 1). All ten produced phenotypically normal adult flies which were genotypically *trx*[−]/+. Hence absence of *trx*⁺ from the germ line does not prohibit normal development, provided the zygote carries a *trx*⁺ allele. This also shows that oogenesis itself does not require the *trx* gene. As expected, since *trx* mutations are zygotic lethal, the remaining animals died on completion of embryogenesis. All the *trx*[−] larvae from the ten *trx*[−] clones analysed were phenotypically similar to *trx*[−] larvae

Table 1 Fertile germ line clones recovered and analysed

Female genotype	No. flies scored	No. fertile clones resulting from:	
		Distal crossover	Proximal crossover
E	753	3	10
C	734	1	13

Experimental females (E) were females genotypically *y v mal f/Dp(3;1)kar*⁵¹, *trx*⁺; *trx*³/*Df(3R)red*^{P93}, *trx*[−]*Sb*, and could therefore carry *trx*[−] clones. Control females (C) were sibs genotypically *y v mal f/Dp(3;1)kar*⁵¹; *trx*³/*TM2* or *Df(3R)red*^{P93}/*Sb TM2*. These females could bear clones having one or two *trx*⁺ alleles. All clone-bearing females were crossed to *y v f mal*; *Df(3R)red*^{P93} *Sb/TM2*, or *y v f mal*; *trx*²/*TM3*, *y*⁺ males. Most clones produced between 50 and 300 eggs. The genotype of every clone except one was determined by analysis of at least 20 adult progeny (Fig. 2). At least 10 unhatched larvae from each clone were mounted for microscopic examination. Amongst progeny of the second cross (four clones) *trx*[−] larvae could be unequivocally identified since they are marked with *yellow* (the *TM3* chromosome carries both *trx*⁺ and *y*⁺).

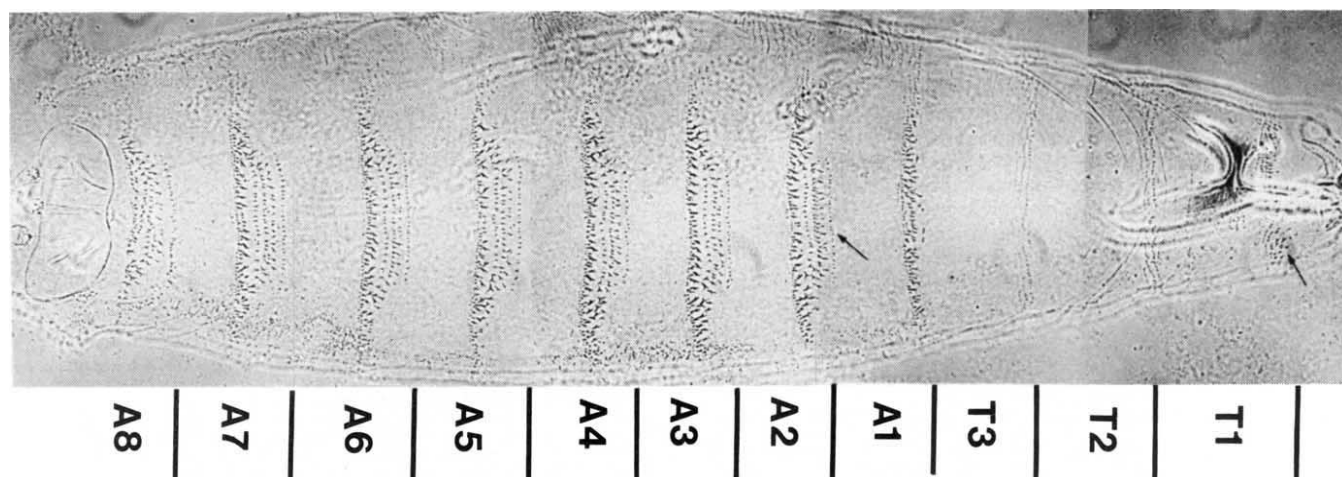


Fig. 1 Ventral cuticle of a *trx*⁻ larva derived from a *trx*⁻ germ line clone. Larvae were mounted in Hoyer's medium either directly or following fixation and clearing by the Van der Meer procedure¹⁴. For a detailed description of the wild-type larval cuticular pattern see ref. 15. Normal differentiation of segment T1 is shown by a band of denticles (arrowed) significantly bigger than those of T2 and T3. The characteristic second mid-ventral patch of denticles is also present in T1. The denticles in segment T3 appear smaller than those of wild type; they resemble those of segment T2 and may indicate a T3 to T2 transformation. Evidence for a transformation of abdominal towards thoracic segments, for instance, the presence of characteristic thoracic structure such as ventral pits and Keilin's organs, is never found. As in wild type, segment A1 differs from the other abdominal segments in lacking an anterior row of anteriorly pointing denticles (indicated by an arrow in A2). These occur in all remaining abdominal segments and each denticle band is considerably broader than that of A1; thus, there is no evidence for transformation of any abdominal segment to A1. The four most posterior segments differ from their wild-type equivalents in the relative widths of their anterior-most and posterior-most denticle rows. The anterior rows tend to be slightly smaller than in wild type; they resemble more those of segments A3 or A4; note that in this larva, as in wild type the denticles of this row in segment A2 are smaller than in the other segments. The transformation of segment A8 is most striking (compare with the A8 patterns in Fig. 3), and is a consistent feature of larvae derived from *trx*⁻ germ line clones. Note, however, the absence of chitinous plates posterior to A8, reported by Duncan and Lewis to be very frequent amongst *trx*⁻ larvae which also lack one copy of the *BX-C*¹¹.

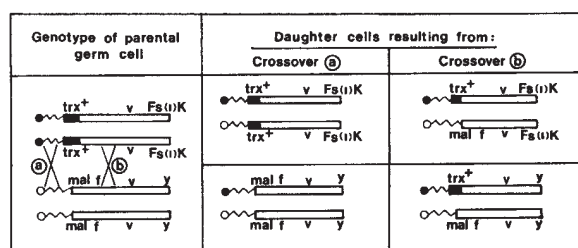


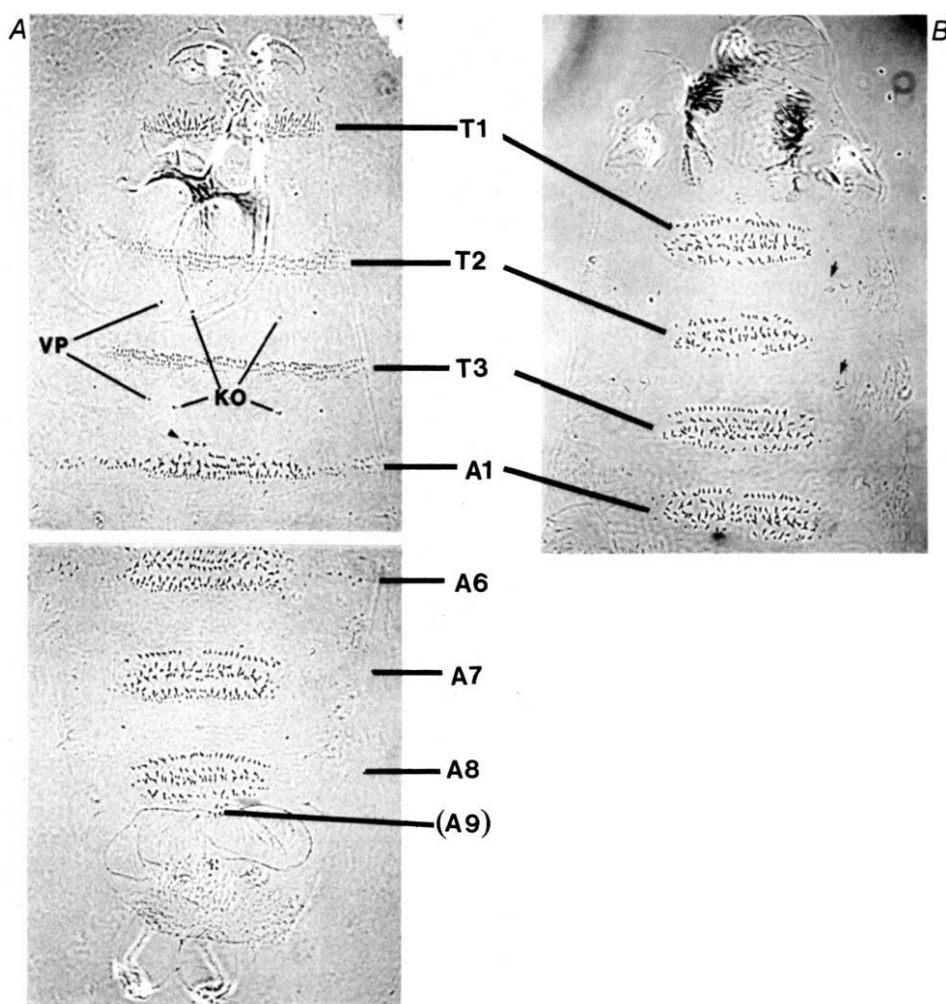
Fig. 2 Production of *trx* clones in the female germ line. An X chromosome carrying a duplication of the *trx*⁺ gene (*Dp(3;1)kar*⁵¹) in *cis* with the dominant female sterile allele *Fs(1)K*¹¹⁰³ was constructed by recombination in females carrying a duplication of the *Fs(1)K*⁺ gene, which covers the dominant sterility of *Fs(1)K*¹¹⁰³ (see ref. 16). First instar larvae from the cross *yvf mal; Df(3R)red*^{P93} *Sb/TM2* × *Dp(3;1)kar*⁵¹ *v Fs(1)K*¹¹⁰³; *red trx*³ were irradiated with 1,000 rad of X rays. (Philips Constant Potential X ray MG102 10 mA 100 kV). Surviving virgin females were collected, crossed (Table 1) and screened for egg production. All females were sterile except where mitotic recombination in a germ cell gave rise to clones lacking the dominant sterile mutation; some of these had simultaneously lost *Dp(3;1)kar*⁵¹ and hence germ line clones lacking a *trx*⁺ gene were produced. The genotype of clones was determined from the genotype of the adult progeny. Progeny of type (a) clones should all be *v f* and *mal* and carry the balancer chromosomes *TM2* or *TM3*. Clones resulting from crossovers distal to *trx*⁺ produce progeny carrying various combinations of the X-linked markers. Hence the two classes of clones can be easily and unequivocally distinguished. References 10 and 17 describe the genetic variants employed. The *Fs(1)K*¹¹⁰³ allele is a germ-line autonomous, agametic, dominant female sterile mutation isolated and characterized by M. Gans and K. Kommitopolou (personal communication).

derived from control *trx*⁻/+ clones or normal *trx*⁻/+ heterozygotes (Fig. 1). Therefore, absence of both the *trx*⁺ gene and its product from the embryo does not prohibit the differentiation of segment specific characteristics. Clearly if *trx*⁺ were essential for the expression of *Scr* and the *BX-C* genes its absence should result in all the thoracic and abdominal segments differentiating a mesothoracic pattern. The phenotype observed is consistent only with absence of *trx*⁺ resulting in sub-optimal expression of the *BX-C* in some segments.

This experiment reveals striking differences between the effects of absence of *trx*⁺ on the development of larval and on adult tissue: for example, the larval prothoracic segment differentiates normally whilst its adult counterpart is transformed to mesothorax¹⁰. This suggests that, as with other putative regulatory genes, for instance, the *Pcl* gene⁸ and *esc* gene^{5,12,13} the requirement for the *trx* gene varies between developmental stages.

Unlike *trx*, the *esc* gene is essential during oogenesis for normal embryogenesis. In *esc*⁻ larvae from *esc*⁻ mothers all abdominal and thoracic and at least two head segments are transformed to eighth abdominal segment (A8), whilst those derived from females carrying an *esc*⁺ allele develop into phenotypically wild-type larvae⁵. Moreover, Struhl⁵ has shown that the segmental transformations caused by *esc* are due to the ectopic expression of the *BX-C* genes. Since absence of the *trx*⁺ gene apparently results in sub-optimal expression of *BX-C* genes, I investigated the effect of removal of *trx*⁺ on the expression of the *esc* phenotype. *esc*⁻; *trx*⁻ zygotes were produced from *esc*⁻ females (Fig. 3). Sibling *esc*⁻ larvae which carry at least one *trx*⁺ allele show the typical *esc* phenotype (Fig. 3B), similar to that described previously. Larvae which are both *esc*⁻ and *trx*⁻ however, exhibit an almost total suppression of the head and thoracic transformations (Fig. 3).

Fig. 3 Suppression of the *esc*⁻ phenotype in *esc*⁻; *trx*⁻ larvae. 201 unhatched larvae from *y*; *esc*⁶/*esc*¹⁰, *trx*³/*TM3*, *y*⁺ parents were prepared as described (Fig. 2) and examined using phase contrast microscopy. Larvae homozygous for *trx*³ could be unambiguously distinguished from their *TM3* (*trx*⁺)-bearing sibs which carry a *y*⁺ allele: all phenotypically *y* larvae are therefore homozygous for *trx*³. A total of 55 *esc*⁻ *trx*⁻ larvae were scored for the following traits: abdominal denticles in the head segments; thoracic denticle belts and sense organs (Keilin's organs, *KO*; ventral pits, *VP*) or abdominal denticles in the thoracic segments; presence of anteriorly pointing denticles in the first abdominal segment, lateral narrowing of abdominal denticle belts, presence of an additional (9th) abdominal denticle cluster; presence of chitinous plates at posterior of larva. The anterior and posterior regions of a typical example are shown in (A). Every larva examined has a normal prothoracic segment (T1); all but five of the larvae also have normal meso (T2) and metathoracic (T3) segments. Exceptionally, a few large denticles characteristic of the abdominal segments are present in T3, and in three cases, T2. In ten larvae segment A1 bears a typical wild-type denticle band. In the remainder an anterior row of anteriorly directed denticles (arrowed) is present. Such a row is characteristic of a more posterior segment, usually it resembles that of A2, A3 or A4 being considerably narrower than the posterior rows of the same belt. The phenotypes of the other abdominal segments vary between larvae, but usually at least some have elements characteristic of segments A6 or A7. There is usually evidence of a 9th denticle belt (A9). Chitinous plates, typical of *trx*⁻ embryos¹¹, were not observed. In most cases, head involution is more or less normal, though parts of the cephalopharyngeal skeleton may be missing. Eleven embryos have abnormal heads with a band of denticles on the dorsal surface similar to that found in *esc*⁻ embryos. The remaining 146 larvae are phenotypically *y*⁺ and therefore carry one or two *trx*⁺ alleles. The denticle bands of all three thoracic segments always resembled that of A8, as shown in (B) though an abnormal organ resembling a Keilin's organ is often present in T1 and T2 (arrowed). None of the larvae showed involution of the head segments. A band of abdominal denticles was always present dorsally in the anterior-most segment whilst in 63 cases similar denticles are present ventrally in the presumptive labial segment. Since *esc*⁻ larvae heterozygous for a *trx*³-bearing and a *Df(3R)red*^{P93} chromosome were phenotypically the same as the *trx*³/*trx*³ larvae, the suppression of the *esc* phenotype cannot be due to some other factor on the *trx*³ chromosome.



Transformation of A1 towards A8 is also suppressed, though this segment still frequently retains some pattern elements characteristic of more posterior segments. The remaining abdominal segments all differentiate patterns which though they cannot be unequivocally assigned to a particular segment type, often resemble those of A6 or A7. Thus expression of the *BX-C* in some abdominal segments reaches a higher level in the absence of both *trx* and *esc* than in the absence of *trx* alone (compare Fig. 1 and Fig. 3). This suggests that removal of either gene has an opposite but independent effect on the expression of the *BX-C*.

Since the thoracic and abdominal segments of *esc*⁻; *trx*⁻ larvae clearly develop differently (Fig. 3), it follows that differential expression of the *BX-C* genes is possible in the absence of the two genes. This implies that regulatory factors other than those coded by the *esc* and *trx* genes, have position specific effects on the expression of the *BX-C*.

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A single gene and a pseudogene for the cellular tumour antigen p53

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The cellular tumour antigen p53 is a protein found in elevated levels in a great variety of transformed cells (reviewed in ref. 1). Overproduction of p53 was observed in cells transformed by a wide spectrum of agents²⁻⁷ as well as in embryonal carcinoma cells^{3,8} and in spontaneous transformants^{9,10}. Although initially described in mice^{2,3,9}, similar p53-like proteins were also observed in cells of other species, including those derived from several human tumours¹¹. In non-transformed cells the protein turns over very rapidly^{12,13} and its levels appear to correlate with cell proliferation^{14,15}. Thus far, very little has been known about the precise nature of the protein and of the corresponding genes. We now provide evidence for the existence of a single functional gene for murine p53 and a processed pseudogene. The predicted amino acid sequence of murine p53 is also presented.

Analysis of p53 by two-dimensional gel electrophoresis usually reveals a family of polypeptides rather than a single species¹⁶. Although all these variations could be due to post-translational modifications, one could postulate the existence of a family of related genes coding for p53. Differential expression of various members of this family could then explain the alteration in amounts¹ and turnover rates^{12,17,18} of p53 manifest in various transformed cell types.

The isolation of p53-specific cDNA clones¹⁹ has made it possible to approach the study of the pertinent genes and mRNA molecules in a direct fashion. The mRNA of p53, when analysed on a denaturing gel, has an approximate length of 2.0 kilobases (kb), including the poly(A) tract²⁰. The sequence of the corresponding cDNA was established by analysing four separate cDNA clones which overlap each other to yield a contiguous sequence of 1,715 base pairs (bp) (see Fig. 1). Three of those clones, all derived from SVT2 mRNA, have been described previously: pp53-208 (ref. 19), pp53-422 and pp53-271 (ref. 20), abbreviated p208, p422 and p271, respectively. A fourth clone, pp53-176 (abbreviated p176), was derived from Meth A mRNA⁹ using the same cloning procedure used for the previously described clones. As discussed below, the available cDNA clones lack the 3' 55 nucleotides of the mRNA. The sequence of these nucleotides was determined from a cloned genomic 3.3-kb fragment, as this fragment was found to represent a processed gene (see below) which has retained the 3' poly(A) stretch 55 nucleotides downstream to the 3' end of clones p208 and p422 (see Fig. 2).

The sequencing strategy is shown in Fig. 1. The sequence as shown in Fig. 2 spans 1,769 nucleotides from the 5' end of p176 to the poly(A) tract found in the genomic 3.3-kb fragment. The identity of the coding strand was established by two experiments: S₁ nuclease analysis, as described before²⁰, and primer extension of the 5'-end labelled *Xho*I-*Hae*III fragment from p176 (nucleotides 41 to -50). This fragment was annealed to IB9 mRNA²⁰, followed by reverse transcription^{21,22}. The major extended band, 155 ± 3 nucleotides in size, was eluted from the gel and sequenced. The sequence of the extended region was complementary to that of nucleotides -51 to -107 shown in Fig. 2. These data confirmed the identity of the coding strand and established the order of the cDNA clones as shown in Fig. 1.

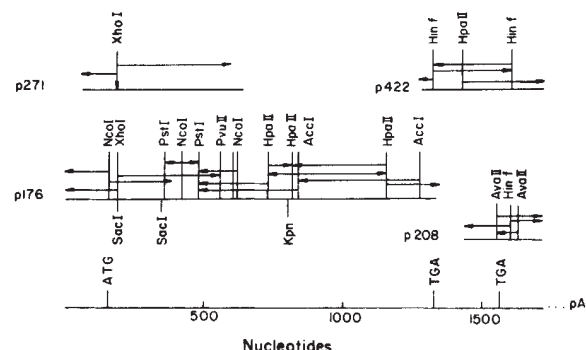
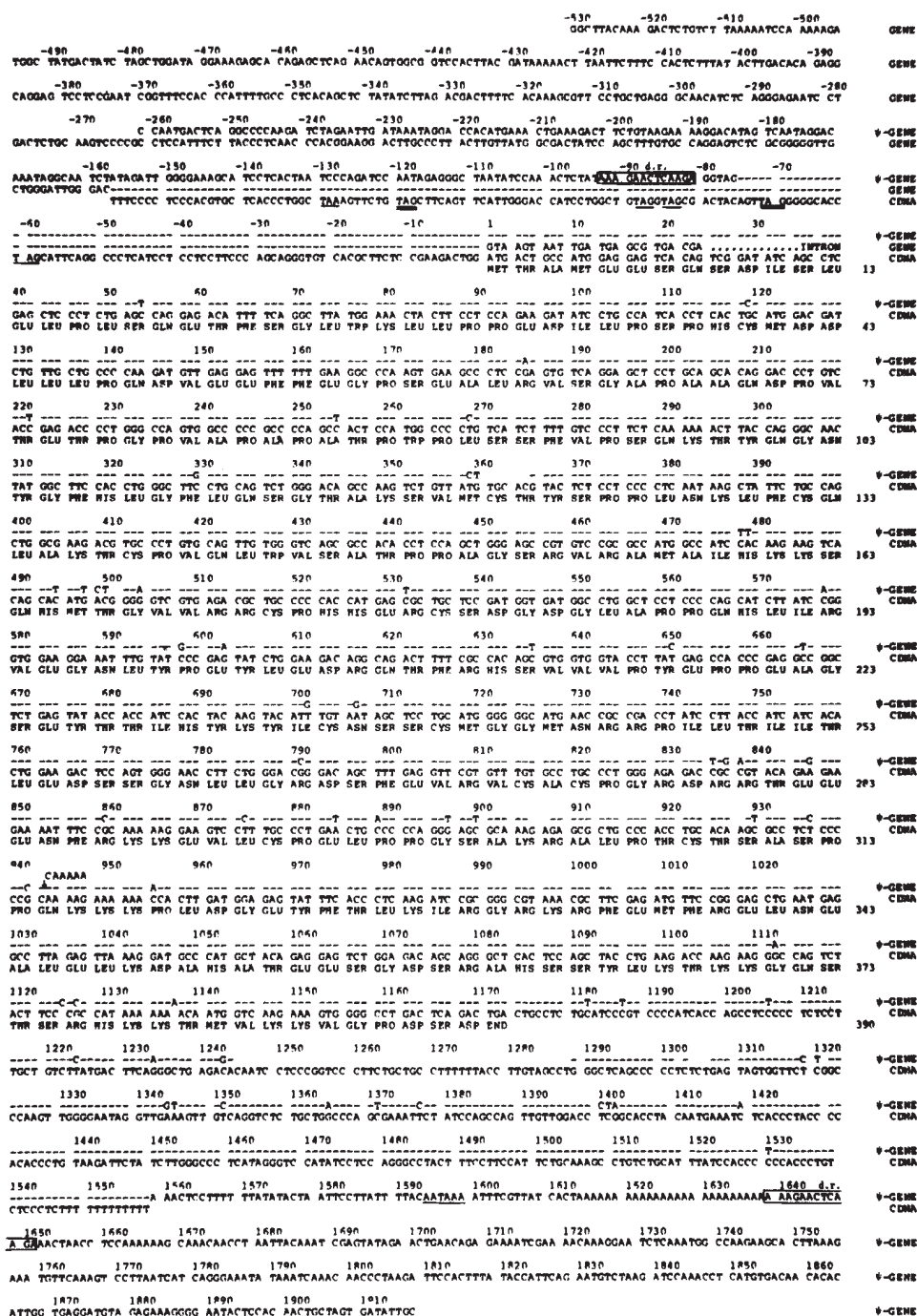


Fig. 1 Restriction enzyme maps and sequencing strategy of p53 cDNA clones. DNA sequence was determined by the chemical degradation method²⁸ of end-labelled fragments generated by the indicated restriction enzymes. 3' End-labelling was done with reverse transcriptase and the appropriate ³²P-deoxynucleotide. The lower line represents the composite structure of the four clones and also includes 55 nucleotides (marked by dots at the 3' end) from the processed gene (see text). The position of the first ATG and two terminators (TGA) is marked. According to the sequence numbering presented in Fig. 2, p176 spans nucleotides -157 to 1,188, p271 spans nucleotides -97 to 480, p422 spans nucleotides 1,129-1,558 and p208 spans nucleotides 1,273-1,558.

Analysis of the sequence data reveals that the only large open reading frame is present between nucleotides 1 and 1,170. Although the existence of cloning artefacts cannot be absolutely excluded at this stage, the main conclusions presented below are strongly supported by several types of evidence. The validity of the reading phase is corroborated by a 17 amino acid sequence from a p53-derived peptide determined by K. Leppard *et al.*³⁰. The assignment of the ATG at position 1 as the first amino acid of p53 is based on the finding that 5' to this codon there are termination codons in all three reading frames. The presence of these terminators (underlined in Fig. 2) was established in both p176 and p271, derived from different cDNA libraries, as well as in the extended primer sequence described above and in the corresponding region of the functional gene (see Fig. 2). Hence, p53 mRNA contains at least 156 nucleotides of 5'-non-coding sequence. The 3' end of the open reading frame is dictated by the presence of a termination codon (TGA) at nucleotides 1,171-1,173; this TGA is found in both p176 and p422, which are independently derived cDNA clones. Overall, mouse p53 is predicted to consist of 390 amino acids, suggesting that its actual molecular weight (43,346) is well below that estimated from SDS gels¹. This apparent difference may be due to some unique properties of p53, such as the high proportion of proline residues (Table 1) and the unusual distribution of these residues along the molecule. For instance, the sequence between codons 70-90 contains seven prolines, and in addition there are seven Pro-Pro pairs (Fig. 2).

Table 1 Amino acid composition of murine p53

Amino acid	No. of residues	Amino acid	No. of residues
Ala	24	Leu	35
Arg	24	Lys	24
Asn	8	Met	10
Asp	17	Phe	13
Cys	12	Pro	38
Gln	14	Ser	35
Glu	31	Thr	24
Gly	25	Trp	3
His	12	Tyr	11
Ile	10	Val	20



Southern blot analysis of BALB/c DNA probed with different cDNA clones has demonstrated the existence of two distinct p53-specific genes in the mouse genome, represented by *Eco*RI fragments of 16 and 3.3 kb, respectively²⁰. Several recombinant phages containing the 3.3-kb fragment were isolated from a BALB/c genomic library²³. In all of these, only the 3.3-kb fragment hybridized to p53 cDNA clones and this fragment was subcloned into pBR322 (pCh53-11) and subjected to DNA sequencing according to the strategy shown in Fig. 3. The sequence of the region between the *Eco*RI and *Bgl*II sites of pCh53-11 (Fig. 3) is shown in Fig. 2 (ψ gene), in parallel with the cDNA sequence. The most striking outcome of this comparison is that the genomic 3.3-kb fragment lacks introns and is almost colinear with the cDNA. The sequences of the cDNA and the ψ gene are almost identical from nucleotide -75 onward. Upstream of this position the two sequences diverge totally and no homology can be observed (Fig. 2). Downstream to

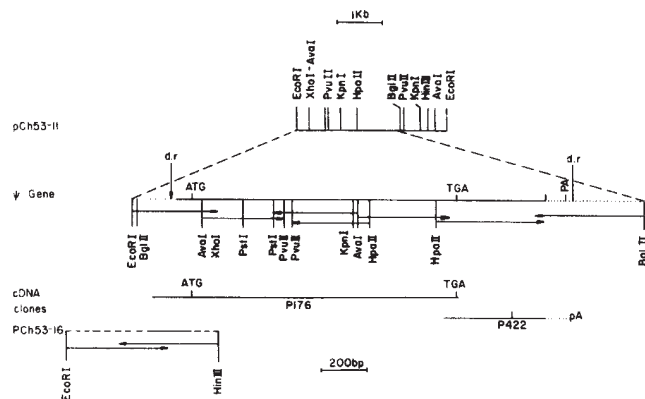


Fig. 3 Restriction enzyme map and sequencing strategy of the processed p53 gene. The figure also shows a comparison of this gene with the cDNA clones and the 5' terminal part of the p53 gene contained in the 16-kb fragment (Ch53-16). —, Sequences present in all clones analysed; ---, sequences present only in Ch53-16; - · - · -, sequences present only in pCh53-11. The 3' end of the mRNA (dotted line next to p422) was deduced from the processed gene as detailed in the text.

nucleotide -75 the two sequences differ by only 4% (61 out of 1,633 nucleotides). This is mostly due to substitutions and to some small deletions or additions in this gene relative to the cDNA.

An interesting feature of the DNA sequence of pCh53-11 is the long (28 bp) poly(A) tract starting at nucleotide 1,614. This poly(A) tract and the absence of introns in this gene suggest that it is a processed gene which resulted from reverse transcription of the mature mRNA²⁴⁻²⁷. Note, however, that neither cDNA clone corresponding to the 3' portion of the mRNA (p208 and p422) contains the copy of the poly(A) tract. Rather, they both end at nucleotide 1,558, which is preceded by an oligo(T). This is likely to be due to self-priming of p53 mRNA by annealing of the poly(A) tract to the T₁₂ tract during reverse transcription.

Another interesting feature of the 3.3-kb fragment is the presence of a 13-bp direct repeat at both ends of the sequence homologous to p53 mRNA. This sequence—AAAGAACT-CAAGA—is located immediately 3' of the poly(A) tract and 5 bp upstream of nucleotide -75, where the p176 and pCh53-1 sequences diverge (boxed in Fig. 2). These six bases, AGGTAG, are, in fact, identical to the cDNA sequence between nucleotides -88 and -83. It is therefore likely that the inserted processed gene underwent a 7-bp deletion (nucleotides -82 to -76) and that the direct repeat is indeed located precisely at the end of the insert. Hence, the genomic clone pCh53-11 possesses three features of processed genes: it is without introns, contains a poly(A) tract and is bounded by direct repeats. Preliminary analysis also suggests that this processed gene is present on a different chromosome from the gene located at the 16-kb fragment and we therefore conclude that the 3.3-kb fragment must contain a processed gene possessing 1,688 nucleotides of the p53 mRNA. As there is a consensus TATA box 5' to the processed gene (nucleotide -96), one might still have considered the possibility that, on transcription, it yields a particular species of p53. This possibility, however, is ruled out on examination of the processed gene nucleotide sequence (Fig. 2), as the deletions at several places will modify the reading frame and introduce terminators within all the reading phases.

The sequence data shown in Fig. 2 indicate that at its 5' end the p53 cDNA contains 82 nucleotides which are absent from the processed gene. This fact, as well as the multiple differences between the sequences of the cDNA and the processed gene, strongly argue against the possibility that p53 mRNA is the product of the latter, implying that the normal gene must be located in the 16-kb *EcoRI* fragment. We therefore compared the 5' cDNA sequence with that of a recombinant phage contain-

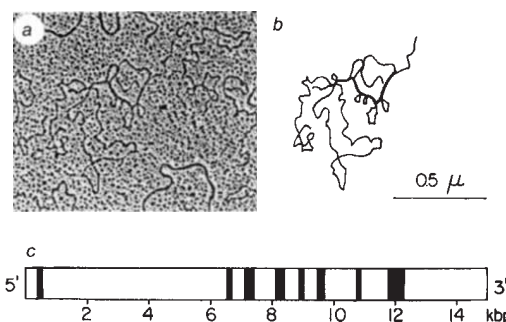


Fig. 4 Heteroduplex analysis of the processed gene (pCh53-11) and the genomic clone Ch53-16. **a**, Electron micrograph of the heteroduplex structure formed between pCh53-11 and the insert of Ch53-16. The insert was isolated on a 0.6% agarose gel and Ch53-11 was linearized with *Bam*HI. Equal amounts (0.06 μ g) of the two DNAs were mixed, denatured at 0.1 M NaOH for 10 min, neutralized by adjusting the solution to 160 mM Tris-HCl pH 8.5 and 0.4 M NaClO₄, and formamide was added to 50% (v/v). Renaturation was allowed to proceed at 37 °C for 1 h and the solution spread on a 10% formamide hypophase. Samples were prepared for electron microscopy (Philips EM 410) by the formamide/cytochrome monolayer spreading procedure²⁹. DNA contour lengths were measured with Φ X174 and simian virus 40 DNA serving as internal length standards for single and double strands, respectively. **b**, Contour scheme of the electron micrograph shown in **a**. **c**, Physical map of the exons (black boxes) and introns were determined from both heteroduplex structures and from restriction map analysis and hybridization with the various cDNA clones. p176 showed hybridization to all eight exons whereas p422 and p208 hybridized only to the last exon and p271 hybridized only to the first four exons. The size of the insert in pCh53-16 was about 15 kb, slightly smaller than the 16-kb estimate determined from genomic blots.

ing this 16-kb fragment (Ch53-16). The sequence of the 5' end of the 16-kb insert was determined as shown in Fig. 3 and is given in Fig. 2 in comparison with the cDNA. The genomic DNA and the 5' end of the cDNA share 157 bp, of which only those downstream of position -75 are also present in the processed pseudogene. Thus, the processed gene must contain a truncated copy of p53 cDNA. The sequence of the gene (Ch53-16) diverges from that of the cDNA at nucleotide -1, exactly before the first ATG, where a typical splice site GGGT is present in the normal gene (Ch53-16), but absent in the processed one (Ch53-11). Hence, this point of sequence divergence probably represents an intron in the p53 gene. To define the structure of Ch53-16, we performed heteroduplex analysis of the *EcoRI* insert of this phage with either p176 or pCh53-11. In both cases, very similar heteroduplex structures were observed, except that the 3' exon in the heteroduplex with pCh53-11 was approximately 400 bp longer than in the one with p176. In both cases at least eight exons could be identified. A typical heteroduplex between pCh53-11 and the insert of Ch53-16 is shown in Fig. 4. Contour measurements of 30 such molecules showed the following sizes (in bp) of the exons (black boxes in Fig. 4) starting from the 5' end: (1) 100 ± 20 ; (2) 110 ± 20 ; (3) 290 ± 50 ; (4) 340 ± 40 ; (5) 130 ± 20 ; (6) 230 ± 40 ; (7) 130 ± 20 ; (8) 500 ± 30 , whereas the last exon was only 130 ± 20 in the heteroduplex between p176 and Ch53-16. The sizes of the introns were as follows: (1) $6,100 \pm 650$; (2) 310 ± 50 ; (3) 750 ± 75 ; (4) 350 ± 40 ; (5) 330 ± 40 ; (6) 900 ± 80 ; (7) 670 ± 90 . These results corroborate the processed gene nature of pCh53-11 and indicate that the p53 gene spans at least 80% of the insert of Ch53-16. The elimination of the processed gene as a possible source of functional p53 mRNA implies that the protein is exclusively encoded by the information present in the 16-kb *EcoRI* fragment. The heteroduplex analysis clearly demonstrates that this fragment contains only a single p53-specific gene; thus, there is only one functional p53 gene in the mouse genome. We therefore conclude that all existing different forms of murine

p53 must be products of the same gene, probably mostly due to post-translational modifications.

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The generation of human monocyte/macrophage cell lines

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The development of continuous murine macrophage-like cell lines has permitted direct and somatic cell genetic approaches to a variety of functions of murine mononuclear phagocytes^{1–3}. However, few, if any, human cell lines exist that have the general characteristics of macrophages, although a promonocyte line, U937, has been reported which can be induced to differentiate into non-dividing cells with macrophage-like properties⁴. Although murine macrophage-like lines have been obtained by transformation with simian virus 40 (SV40)^{5,6}, this technique has not, in general, been useful in producing human cell lines, presumably because SV40 causes a lytic infection in human cells. We have therefore developed a method for transfecting primary human monocytes with SV40 DNA deleted in the origin of replication. Using this method, we have now, we believe for the first time, generated transformed human cell lines with macrophage characteristics. Three cell lines from three transfections of human monocytes expressed macrophage enzymes, phagocytic function, surface receptors including HLA-DR and DS and alloantigen presenting activity.

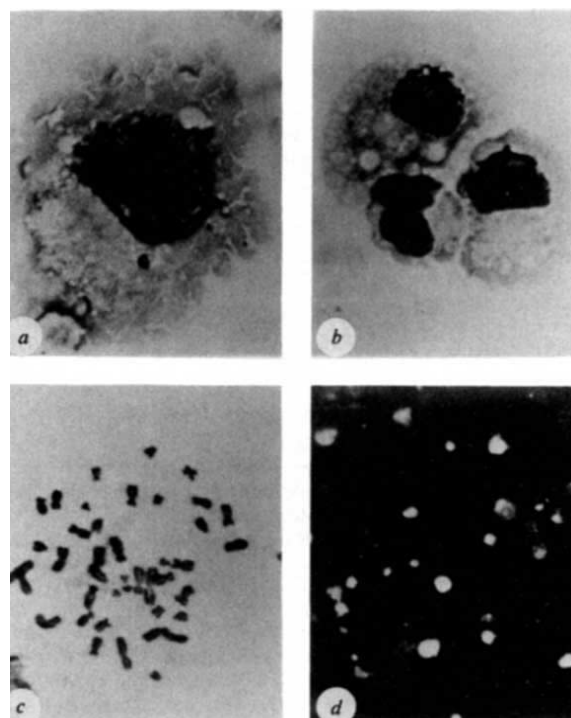


Fig. 1 Cytological characteristics of: *a*, line DM; *b*, line BB. Giemsa stain ($\times 1,000$). *c*, The karyotype of line BB, displaying chromosomes of human origin. Giemsa stain ($\times 1,000$). *d*, SV40 T antigen expression of nuclei from line DM ($\times 200$). Nuclei were extracted with 0.5% NP40 and stained with polyclonal rabbit anti-SV40 T antigen, followed by fluoresceinated goat anti-rabbit immunoglobulin.

Origin-defective SV40 DNA (SV40 ori⁻) was initially provided by Dr J. Sambrook and used to transform *Escherichia coli* using calcium phosphate precipitation^{7,8}. The isolation of covalently closed circular DNA was accomplished using ethidium bromide–CsCl gradients, and molecular sieve chromatography was used to separate DNA from RNA^{9,10}.

Peripheral blood cells were obtained by venepuncture from healthy volunteers and mononuclear cells were separated by Ficoll–Hypaque sedimentation¹¹. A calcium phosphate precipitate formed with SV40 ori⁻ DNA (30–50 μ g) was added to primary mononuclear cells ($1\text{--}2 \times 10^7$ cells in 20 ml of serum-free medium), centrifuged at 1,500 r.p.m. for 5 min. To facilitate uptake of the DNA, 50% polyethylene glycol (molecular weight 4,000) and 5% dimethyl sulphoxide in fusion salts (0.14 M NaCl, 5.4 mM KCl, 10 mM Na₂HPO₄, 5 mM NaH₂PO₄, 0.2% (w/v) glucose, pH 7.2) were added to the pellet and incubated for 90 s at 37 °C followed by immediate dilution with 20 ml of the above salts¹². The cells were washed, resuspended in medium consisting of Dulbecco's modified Eagle medium (DME), 20% heat-inactivated fetal calf serum, 10% NCTC 109 medium (Microbiological Associates), 0.1 mM non-essential amino acids (Gibco), 2 mM glutamine, penicillin and streptomycin and 5% colony stimulating factor (CSF, Gibco), specific to granulocytes and macrophages, plated in 2 cm² wells (Linbro) and cultured in a 37 °C incubator. Medium was changed at least once a week. Within 1–2 months after transfection, transformed lines from three independent transfections with SV40 ori⁻ DNA were obtained. (Primary cells cultured in this medium, but not transfected, survived no longer than 1 month.)

All lines obtained in this fashion have the phenotype of macrophages. The morphology of these three lines, DM, BB and CS, resembles that of primary human macrophages (Fig. 1*a, b*). Their karyotypes, including G-banding, display 38–51 human chromosomes per cell (Fig. 1*c*)¹³. Nuclei from these lines revealed the presence of SV40 T antigen by immuno-

Table 1 The characteristics of cell lines

Cell line	DM	BB	CS
Nonspecific esterase	(+)	(+)	(+)
Peroxidase	Slightly (+)	Slightly (+)	Slightly (+)
Lysozyme (per 10 ⁶ cells per 24 h)	5 µg	5 µg	5 µg
Collagenase (per 10 ⁷ cells)	18.8 µg	36.0 µg	20.4 µg
Fc receptors	52%	58%	44%
Fc phagocytosis	42%	42%	20%
Complement receptors	98%	90%	52%
Complement phagocytosis	48%	10%	11%
DR determinants	51.2%	46.0%	39.1%
DS determinants	35.3%	32.8%	21.1%
IL-1 production (per 10 ⁶ cells per 72 h)	1.4 U	1.8 U	0.9 U
(induced with LPS)	3.2 U	3.4 U	1.3 U

Secretion of lysozyme was determined by a lysoplate assay which measures lysis of *Micrococcus lysodeikticus* in agar using hen egg white lysozyme as a standard²⁰. Collagenase was determined by measuring degradation of ³H-collagen (NEN) by each cell extract, using tadpole collagenase (NEN) as a standard. The presence of Fc receptors and Fc mediated phagocytosis was analysed by rosette formation with rabbit IgG-coated sheep red blood cells (SRBCs)^{21,22}. Rosettes were defined as a cell binding 3 or more immunoglobulin-SRBCs. To test for complement receptors and phagocytosis, SRBCs were incubated with rabbit IgM anti-SRBC antibody and fresh BALB/c mouse serum as a source of complement. Rosetting and phagocytosis were assayed as for IgG-coated SRBCs. A monoclonal mouse IgG anti-human DR antibody was used and fluoresceinated rabbit anti-mouse IgG was used as a second antibody. To stain DS determinants of Ia molecules, a rabbit serum directed against human DS determinant, Rb03, was used, followed by fluoresceinated goat anti-rabbit immunoglobulin. Cells were analysed on a fluorescence-activated cell sorter (FACS II). To detect IL-1 secretion, the supernatants from 3-day culture fluid of 10⁶ cells ml⁻¹ with or without 10 µg ml⁻¹ LPS were added to thymocytes prepared from 4–6-week-old C3H/HEJ mice. After 72 h incubation at 37 °C, thymocyte proliferation was determined by ³H-thymidine incorporation. Media containing 5% CSF were negative in the lysozyme, collagenase and IL-1 assays.

fluorescence with a rabbit polyclonal anti-T antigen and a fluoresceinated goat anti-rabbit IgG (Fig. 1d). All cell lines showed histochemical properties of monocytes, and all secreted lysozyme and collagenase (Table 1). As also shown in Table 1, the DM line is 52% positive for Fc receptors and 42% positive for Fc receptor mediated phagocytosis, the BB line is 58 and 42% positive, respectively, and the CS line 44 and 20%, respectively. All were more than 50% positive for complement receptors and demonstrated complement receptor mediated phagocytosis.

Using the fluorescence analyser, surface antigen expression was examined with the following antibodies: OKM1, a macrophage marker¹⁴; Leu7, a natural killer cell marker¹⁵; OKT3, a pan-T-cell marker; polyvalent anti-human IgM and IgG for detecting surface immunoglobulin; a murine monoclonal anti-DR antibody; and a rabbit anti-DS antiserum^{16,17}. Cells were OKM1 positive, Leu7 negative, OKT3 negative and surface immunoglobulin negative. All lines expressed DR and DS determinants. In addition, all lines can be lysed by anti-DR antibody and complement, confirming the presence of DR antigens on the membrane (Table 2). Furthermore, both the DM and BB lines stimulate a mixed lymphocyte culture (Table 3). Because T antigen is thought to be the unique viral protein on the membrane of cells transformed with SV40 DNA¹⁸, it was important to show that allogenic T cells were not proliferating in response to T antigen rather than in response to Ia molecules. As can be seen in Table 3, antibody to T antigen did not block the cells' stimulatory activity, while anti-Ia (anti-DR) antibodies did inhibit the proliferation of allogenic T cells.

Finally, the lines were assayed for interleukin-1 (IL-1) production. Although they produce only small amounts of IL-1

Table 2 Complement-mediated lysis of DM, BB and CS cells

		% Lysis	
		-C'	+C'
DM	—	22	23
	Anti-DR	11	80
	Irrelevant antibody	12	23
BB	—	29	33
	Anti-DR	22	82
	Irrelevant antibody	21	27
CS	—	21	23
	Anti-DR	13	82
	Irrelevant antibody	18	23

Cells were incubated with either mouse monoclonal anti-DR antibody or an irrelevant antibody at 4 °C for 30 min, followed by treatment with rabbit complement (Cedarlane Laboratories) at 37 °C for 1 h. Lysed cells were counted using trypan blue dye exclusion. 200 cells were counted in duplicate assays.

Table 3 Mixed lymphocyte culture

		Responder (c.p.m.)		
a	Stimulator	CG	AL	—
	DM	12,359	9,817	693
	+ anti-DR	4,032	5,602	594
	+ anti-TAg	10,528	9,707	649
	—	1,926	760	—
b	Stimulator	AL	DM	—
	BB	35,938	21,224	2,313
	+ anti-DR	5,466	7,580	2,297
	+ anti-TAg	31,177	25,980	1,607
	—	1,350	631	—

2 × 10⁵ primary mononuclear cells from healthy volunteers were incubated with 2 × 10⁴ irradiated DM or BB cells (1,000 rads) for 4 days. Cell proliferation was determined by ³H-thymidine incorporation. Transformed cells were also treated with anti-DR antibody and/or a polyvalent anti-SV40 T antigen (anti-TAg) for 30 min at 4 °C, washed extensively, plated and compared with untreated transformed cells. CSF was removed from the medium and each assay consisted of triplicate cultures. In a, 2 × 10⁵ primary cells from donor CG cultured with the same number of primary cells from AL was 8,348 c.p.m. In b, the same number of primary cells from donor AL cultured with donor DM was 25,272 c.p.m.

constitutively, their production of IL-1 can be enhanced by lipopolysaccharide (LPS) induction (Table 1).

The technique of fusing SV40 ori⁻ DNA to primary monocytes induced to proliferate with monocyte-specific growth factors can be used to generate functional mature human macrophage cell lines. Transformation with SV40 ori⁻ DNA is probably an effective method because this DNA is unable to produce a lytic viral infection. Small *et al.*¹⁹ have previously shown that SV40 ori⁻ DNA transforms human fibroblasts more efficiently than does wild-type DNA, presumably for the same reason. The use of polyethylene glycol and dimethyl sulphoxide in this protocol may drive the DNA into cells which are then stimulated to divide with monocyte-specific growth factors. This protocol therefore satisfies the two requirements for a successful transfection: that DNA enters the cytoplasm and that the cell undergoes at least one cycle of DNA replication. A panel of human monocyte lines obtainable in this manner should be extremely useful in elucidating monocyte heterogeneity and the molecular basis for macrophage function in the immune response.

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Mitochondria control expression of a murine cell surface antigen

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Maternally transmitted antigen (Mta) is a murine cell-surface molecule defined by the reactivity of specific H-2 nonrestricted cytotoxic T lymphocytes (CTL-s)^{1,2}. Maternal transmission appears to be under control of a stable genetic factor in the cytoplasm of the ovum^{3,4}. In view of the known maternal inheritance of mitochondria⁵⁻⁷, we have assessed their involvement in Mta expression using the mitochondria specific poison Rhodamine 6G (R6G)^{8,9}. We report here that Mta expression in somatic cell hybrids requires functional mitochondria from the Mta⁺ parent cell line. Mta expression was dominant in hybrids from the fusion of Mta⁺ and Mta⁻ cells. However, pretreatment of the Mta⁺ parent with R6G resulted in hybrids which were Mta⁻, or diminished in Mta expression. These data strongly implicate mitochondrial DNA (mtDNA) in the expression of a cell-surface molecule, and define a system for studying a previously unrecognized mitochondrial function. To our knowledge, this is the first evidence for mitochondrial control of the expression of a cell membrane molecule in eukaryotes.

Mta is unique in its non-mendelian pattern of inheritance. The Mta phenotype of any mouse is determined solely by the phenotype of the mother; the father has no influence on transmission of Mta¹. Most inbred strains of mice are Mta⁺, with the exception of certain substrains of NZB¹. Thus, (NZB/BINJ δ $\times$ B10.BR δ)F₁ mice are Mta⁻, but the reciprocal (B10.BR δ $\times$ NZB/BINJ δ)F₁ mice are Mta⁺. Sex-linked transmission is ruled out because the phenotypes of F₁ males and females in a single litter are identical¹. Mta phenotype cannot be modified by fostering, embryo transfer or transfer of bone marrow cells to lethally irradiated mice^{3,4}, making the involvement of a conventional infectious agent unlikely. This interpretation is further supported by the finding that the maternal lineage of the Mta⁺ phenotype is stable for at least 11 generations of backcrossing to Mta⁻ males⁴. Analysis of restriction endonuclease (RE) fragment patterns has demonstrated a highly conserved mtDNA genome among the old inbred strains of mice¹⁰⁻¹². Recent

studies conducted in our laboratory¹³, and that of Ferris *et al.*¹⁴, revealed unique mtDNA in those strains which are Mta⁻. Although these results were consistent with involvement of mtDNA in Mta expression, coincidental maternal transmission of both mtDNA and Mta phenotype could not be ruled out.

To test the hypothesis that mtDNA is involved in the control of Mta expression, we asked whether the mitochondrion-specific toxin R6G^{8,9} could prevent expression of Mta in somatic cell hybrids. Mta expression by the hybrids was determined by their susceptibility to lysis by long-term Mta-specific CTL lines. Only cells expressing Mta can be lysed by these CTLs. We have used such CTL lines to type X63-Ag8.653, an 8-azaguanine resistant plasmacytoma of BALB/c origin¹⁵, as Mta⁺. X63-Ag8.653 cells were cultured in R6G for 48 h. The cells were then collected, washed and fused with normal NZB/BINJ splenocytes, and plated in microtitre trays. Control cultures with untreated X63-Ag8.653 cells fused with NZB/BINJ splenocytes were plated at the same time. Hybrids were selected by culturing in medium containing hypoxanthine, aminopterin and thymidine. As reported by Ziegler and Davidson⁹, R6G pretreatment had little effect on the generation of hybrids, although almost 100% of the treated, but unfused X63-Ag8.653 cells died after a few days of culture in the absence of any further R6G. After 2 weeks, a number of cultures were chosen for expansion and subsequently assayed for Mta expression. Of the 10 hybrids tested which were derived from the control fusion with untreated X63-Ag8.653 cells, all were Mta⁺; 5 are shown in Fig. 1. In contrast, 76% (23/30) of those hybrids derived from the R6G-treated X63-Ag8.653 parent failed to express Mta. Nine such lines, shown in Fig. 1, were not lysed by the Mta-specific CTL line 77G11, and one (H2.6) was lysed at a barely detectable level. Those hybrids from the R6G-treated parent that expressed Mta (for example, H3.2 in Fig. 1B) usually did so at a lower level than those from the control fusion. Partial expression of Mta by some of these hybrids was expected, because the R6G treatment may not have completely inactivated all mitochondria contributed by the X63-Ag8.653 parent. Identical typing results have been obtained using five other CTL lines and a mixed population of Mta-specific CTLs from a secondary mixed lymphocyte culture, and the Mta phenotype of the hybrids has remained stable for over 3 months in culture (R.S., unpublished observations).

The possibility that the CTL lines recognized an antigen other than Mta on the hybrids was ruled out by four criteria. First, in two pairs of reciprocal F₁ crosses, only those target cells from the Mta⁺ mice were lysed (Fig. 2A). Second, lysis of target cells from the C57BL/6 (H-2^b), C58 (H-2^k) and B10.S (H-2^s) strains in Fig. 2A demonstrated that the recognition of Mta by the CTL line 77G11 was H-2 nonrestricted; that is, the CTLs do not require simultaneous recognition of Mta and syngeneic molecules encoded by the major histocompatibility complex (MHC). Third, cold target inhibition experiments demonstrated that the same antigen was recognized on all of these targets (for details, see Fig. 2B legend). Finally, the presence of Mta on the hybrids from the untreated control fusion was proven by the complete inhibition of lysis of ⁵¹Cr-labelled (B10.BR δ $\times$ NZB/BINJ δ)F₁ targets (Fig. 3). Failure of the hybrids tested from the R6G-treated fusion partner to inhibit Mta-specific lysis confirms that they were Mta⁻.

To confirm that the R6G pretreatment did not influence the expression of nucleus-encoded cell-surface antigens, we determined that the hybrids did express Qa-1.2, a cell-surface antigen encoded in the MHC of BALB/c, but not NZB¹⁶ (Table 1). All hybrid cell lines were lysed by the Qa-1.2-specific CTL line 27.21-5C6H8¹⁶, although only those hybrids from the control fusion were lysed by the Mta-specific line. Because NZB spleen cells do not express the Qa-1.2 antigen, we concluded that its expression by the hybrids was contributed by the R6G-treated X63-Ag8.653 parent cells.

R6G pretreatment of the Mta⁺ fusion partner prevented, or markedly decreased, Mta expression in daughter hybrid lines. R6G is a highly lipophilic dye known to bind tightly to mitochon-

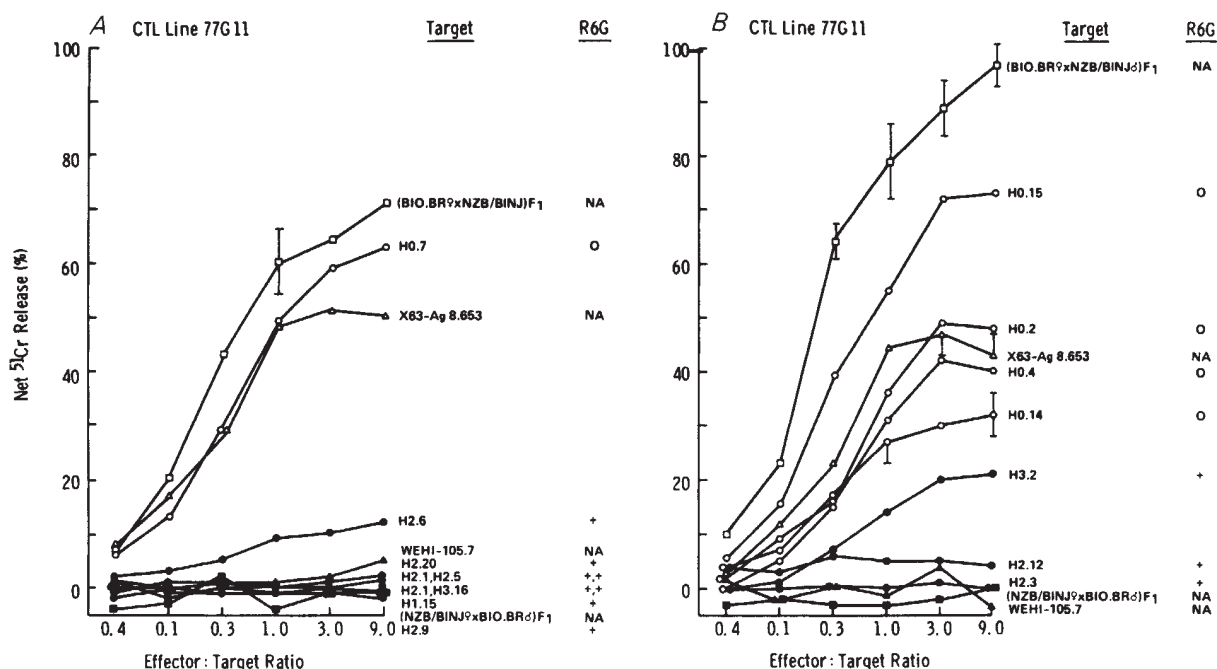
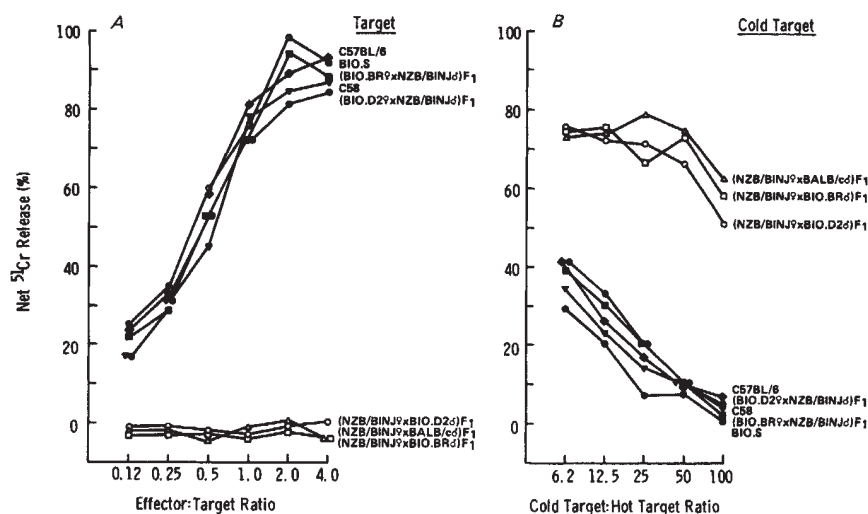


Fig. 1 Sensitivity of hybrid cell lines and controls to lysis by CTL line 77G11. Panels A and B represent two independent assays.

Methods: X63-Ag8.653 cells were either treated for 48 h with R6G (Kodak), at $1 \mu\text{g ml}^{-1}$ in Dulbecco's modified Eagle medium, or left untreated, and then hybridized with normal NZB/BINJ splenocytes by polyethylene glycol mediated fusion. Hybrid cells were selected in hypoxanthine-aminopterin-thymidine medium, expanded and assayed for Mta expression. The hybrid cell lines, as well as the Mta-positive and -negative control cell lines X63-Ag8.653 and WEHI-105.7 (an Mta $^{-}$ thymic leukaemia of NZB/BI origin), were labelled with $150 \mu\text{Ci Na}_2^{51}\text{CrO}_4$ for 1 h at 37°C , washed and plated at 10,000 cells per well in microtitre plates. Lymphoblasts recovered from 48 to 72 h cultures of concanavalin A (Con A) stimulated spleen cells from reciprocal F_1 mice were collected, labelled with $150 \mu\text{Ci Na}_2^{51}\text{CrO}_4$ for 2 h at 37°C , and plated at 10,000 cells per well. CTL line 77G11 was established and maintained by repeated stimulation of (NZB/BINJ $\times$ B10.D2 δ) F_1 splenocytes with irradiated BALB/c spleen cells and a source of interleukin 2, as previously described¹⁶. The line is Thy-1.2 $^{+}$, Ly-1 low and Ly-2 high , as determined by flow cytometry (R.S., unpublished observations). The CTL line was adjusted to 9×10^5 viable cells per ml, and $100 \mu\text{l}$ of threefold dilutions plated in triplicate. After a 4 h incubation at 37°C , the supernatants were collected with the Titertek Supernatant Collection System (Flow). Per cent net ^{51}Cr release was determined by the formula [(experimental release - spontaneous release)/(maximum release - spontaneous release)] $\times 100$, where the spontaneous release is the activity released by target cells in medium alone, and the maximum release is the activity released by target cells submitted to three cycles of freezing and thawing. The spontaneous release varied from 8 to 33% of the maximum release for each target cell in panel A, and from 13 to 31% in panel B. The vertical bars represent 1 s.e. of the % net ^{51}Cr release, however, those less than 2.5% are not shown. The right-hand column of each panel indicates whether X63-Ag8.653 was pretreated with R6G before the fusion from which the hybrid was derived. NA, not applicable.

Fig. 2 A, Con A lymphoblasts were prepared and tested for susceptibility to lysis by the CTL line 77G11, as described in Fig. 1. The spontaneous release varied from 17 to 27% of the maximum release, and the s.e. of the % net ^{51}Cr release did not exceed 6% for any of the triplicate determinations. In B, 77G11 was assayed for ability to lyse (B10.D2 δ $\times$ NZB/BINJ) F_1 ^{51}Cr -labelled lymphoblasts in the presence of the indicated unlabelled target cells. Unlabelled target cells inhibit the lysis of ^{51}Cr labelled target cells only if both express the same antigen recognized by the CTL. No inhibition would be observed if the CTL recognized different antigens on the labelled and unlabelled target cells. The effector to target ratio was 1:1. Unlabelled target cells were prepared exactly like the hot target cells, except that they were not labelled with $\text{Na}_2^{51}\text{CrO}_4$. The spontaneous release was 23% of the maximum release, and the s.e. of the % net ^{51}Cr release was less than 5% for all triplicate determinations. The net ^{51}Cr release in the absence of cold target cells was $72(\pm 5)\%$. In both panels, Mta $^{+}$ target cells are indicated by closed symbols, and Mta $^{-}$ target cells by open symbols.



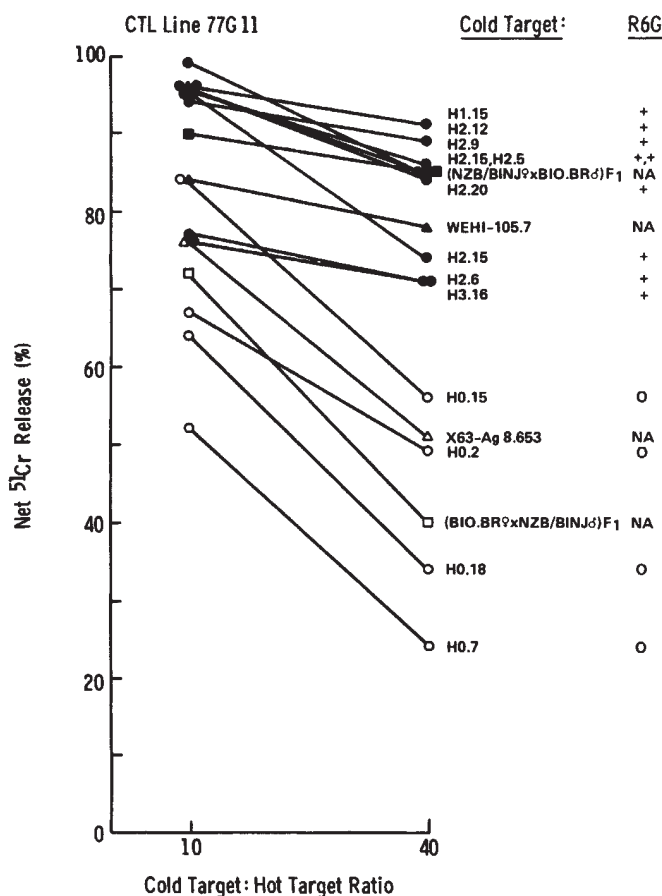


Fig. 3 Hybrids and Con A lymphoblasts were prepared as described in Fig. 1, but not labelled with $\text{Na}_2^{51}\text{CrO}_4$. The hot target cells were from (B10.BR $\times$ NZB/BINJd) F_1 Con A lymphoblasts, labelled with $250 \mu\text{Ci Na}_2^{51}\text{CrO}_4$ for 2 h at 37°C . The labelled blasts were plated at 10,000 cells per well in microtitre wells, with 24,000 77G11 cells and unlabelled target cells at the indicated cold-to-hot target ratio. The spontaneous release was 15% of the maximum release, and the s.e. of the % net ^{51}Cr release did not exceed 5% for any of the triplicate determinations. The net ^{51}Cr release in the absence of cold target cells was $85(\pm 5)\%$.

drial inner and outer membranes, and to block oxidative phosphorylation⁸. In mammalian cells, chloramphenicol (CAP) resistance is controlled by mtDNA¹⁷. In accord with these observations, Ziegler and Davidson⁹ have shown that R6G pretreatment of CAP resistant cells prior to fusion with CAP sensitive cells significantly reduced the frequency of CAP resistant hybrids. Thus, R6G prevented the expression of mtDNA from the treated fusion partner in somatic cell hybrids. Because R6G does not interact directly with mtDNA, but inhibits oxidative phosphorylation, the inhibition of mtDNA expression is a secondary effect of the loss of mitochondrial function, and not due to a direct action of R6G on the mtDNA.

Although it remains a formal possibility, it is highly unlikely that the R6G treatment interfered with the expression of extramitochondrial sources of DNA, such as plasmid or viral DNA. Such genomes are not known to be associated with membranes or to be linked to energy-yielding processes. Even a hypothetical membrane-associated coding element would probably not be sensitive to R6G, because the known action of R6G is its inhibition of oxidative phosphorylation, possibly by blocking the function of the ADP-ATP translocase⁸. Thus, based on its known effects, R6G appears to be specific in its action on mitochondria. Taken together with our results, these observations strongly imply a role for mtDNA in Mta expression.

The data do not suggest a mechanism by which mtDNA might affect Mta expression. Mta shares certain characteristics with

Table 1 Hybrid cell line expression of the Qa-1.2 antigen contributed by X63-Ag8.653

Hybrid	R6G treatment	CTL lines	
		CTL 77G11 (Mta-specific)	CTL 5C6H8 (Qa-1.2-specific)
H0.4	0	$43 \pm 3^*$	26 ± 2
H0.7	0	53 ± 2	39 ± 4
H0.15	0	46 ± 3	39 ± 3
H1.15	+	-3 ± 1	32 ± 1
H2.1	+	0 ± 1	43 ± 2
H2.3	+	-1 ± 1	40 ± 2
H2.5	+	0 ± 1	36 ± 3
H2.9	+	-1 ± 1	29 ± 1
H2.15	+	0 ± 0	35 ± 6
H3.16	+	-1 ± 0	45 ± 2

Hybrids were assayed for susceptibility to lysis by the indicated CTL lines. Effector-to-target ratios were 3:1 for 77G11, and 8:1 for 5C6H8. Spontaneous release values varied over 8–16% of the maximum release. The Qa-1.2 specificity of 27.21-5C6H8 has been demonstrated by its ability to lyse target cells from C57BL/6, but not from the congenic B6-Tla^a strain (R.N.J., unpublished observations).

* Data are expressed as the % net ^{51}Cr release ± 1 s.e. of quadruplicate determinations.

the class I glycoproteins encoded in the MHC, in that both Mta-specific and class I alloantigen-specific CTLs are not restricted for concomitant recognition of syngeneic class I molecules^{1,2}, and both appear to require β_2 -microglobulin for transport to the cell membrane¹⁸. At least 35 class I genes have been identified on chromosome 17 of BALB/c^{19,20}, far more than the number of known class I gene products. Thus, Mta might be encoded by a structural gene in the MHC, and its expression controlled by a mtDNA gene product. A second possibility, that the structural gene for Mta is mitochondrial, is suggested by the finding that some differences between the mtDNA of NZB/BINJ and the old inbred strains map to five unidentified reading frames in the mtDNA^{14,21} (M.M.H., unpublished observations). The Mta gene might be an example of 'promiscuous' DNA, that is, a nuclear gene that has been transported to mitochondria. Several recent reports have identified nucleotide sequences occurring in more than one of the membrane-bound organellar genetic systems of eukaryotes^{22,23}.

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Alternative RNA splicing in expression of the *H-2K* gene

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A major role of the classical transplantation antigens (designated class I antigens) is the presentation of virus-infected cells to cytotoxic T cells, a process that leads to the destruction of the cell displaying the viral antigen¹. Consistent with this function is the finding that these transplantation antigens (encoded by the *H-2K*, *H-2D* and *H-2L* genes in mice) are cell-surface glycoproteins with their amino-termini protruding extracellularly and their carboxy-termini located inside the cell^{2,3}. While the external domain is expected to provide biological specificity required for the associative presentation of viral antigens, the role of the cytoplasmic domain remains obscure. The recent observation that this latter region of the molecule is encoded by three separate DNA exons⁴⁻⁹ has suggested a complex role for this portion of the polypeptide chain. We have now obtained evidence for the use of alternative acceptor splice sites in the *H-2K* gene, resulting in two RNA transcripts that would encode *H-2K* antigens differing in their carboxy-termini. This is the first demonstration of the use of alternative splice acceptor sites in the same class I gene, and indicates the existence of different functional subsets of antigens encoded by the same gene.

In our analysis of mouse class I cDNA clones, we have observed that while transcripts from different members of this multigene family are closely related in their coding sequences, they can be distinguished by their 3' noncoding sequences¹⁰. One such 3' noncoding probe identifies specifically the *H-2K* gene¹¹ and has been used to detect the expression of *H-2K* transcripts in different tissues¹².

Using this criterion, seven cDNA clones derived from liver RNA of SWR mice (*q* haplotype) have been typed as *H-2K*, as distinguished from transcripts from the *H-2D*, *H-2L* and *Qa* regions of the major histocompatibility complex¹¹. The present study compares two of these *H-2K* cDNA clones (pH8 and pH13) (Fig. 1). When plasmid DNA from the two clones was digested with *Pst*I, fractionated by agarose gel electrophoresis, blotted on a nitrocellulose sheet, and hybridized against a ³²P-labelled *H-2K*-specific noncoding probe, the 3'-terminal *Pst*I fragment (~200 base pairs, bp), beginning 239 nucleotides after the termination codon and ending shortly after the polyadenylation signal, was detected in both clones (Fig. 1a). The slight difference in fragment size is due to a difference in the length of their G·C tails which were added to facilitate insertion of the double-stranded cDNA molecules into pBR322 DNA at the *Pst*I site¹³. The specificity of this hybridization confirmed the relatedness of cDNA clones pH8 and pH13 and their assignment to the *H-2K* gene.

Had these two clones been derived from the same mRNA species, the internal *Pst*I fragment, lying immediately upstream from the ~200-bp 3'-terminal fragment, should be identical in size. However, when a ³²P-labelled probe was used to assess this possibility, clone pH8 was found to have a ~575-bp fragment and clone pH13 a ~550-bp fragment (Fig. 1b). The internal *Pst*I fragment, by analogy to our prototype full-length *H-2K* cDNA clone (pH22), is expected to extend from amino acid 237 to a position 239 bp downstream from the termination codon. Further confirmation that clones pH8 and pH13 are both derived from the *H-2K* gene and differ in the size of their internal *Pst*I fragment comes from Southern blot analysis performed using a synthetic octadecadeoxyribonucleotide probe

specific to the *H-2K* gene. (This unique coding probe detects a sequence within the transmembrane domain of the *H-2K* gene at amino acids 295–300 which differs from the analogous region of the *H-2L* and *H-2D* genes at 8 and 7 out of 18 positions, respectively¹¹.) The ³²P-labelled synthetic probe detected both the ~575-bp fragment in clone pH8 and the ~550-bp fragment in clone pH13 (Fig. 1c).

The differences observed between the two *H-2K* cDNA clones may be explained either by the deletion of a short sequence in pH13 which was derived from the same gene as pH8, or by the expression of two separate genes which by two criteria have been identified as *H-2K*. To resolve this question, we analysed the DNA sequence of the two cDNA clones.

Figure 2a presents the sequence of pH8 and pH13 from amino acid 237 to the end of the mRNA, 454 nucleotides downstream from the termination codon. Comparison of the two cDNA clones indicates that no base substitution occurs over a stretch of 780 nucleotides, except for a contiguous 27-base deletion in pH13 relative to pH8 (Fig. 2b). This deletion coincides precisely with sequences from the beginning of exon 8 which encodes the third cytoplasmic domain (I3)⁶⁻⁹.

The perfect conservation of sequences between pH8 and pH13, other than the short deletion, makes it highly unlikely that the two cDNA clones were derived from mRNAs transcribed from two distinct genes. In addition, when compared with either the *H-2K^d* (ref. 8) or *H-2K^b* (ref. 9) sequences, pH8 and pH13 show several unique substitutions in the extracellular region of the molecule. For example, Glu 268 and Thr 280 found in *H-2K^d* and *H-2K^b* have been replaced by Lys 268 and Ala 280, respectively. Whether they represent allelic substitutions specific to *H-2K^a* is unclear, although clearly both substitutions are conserved between pH8 and pH13. Furthermore, Lys 308 in *H-2K^d* and *H-2K^b* is deleted in both pH8 and pH13.

An alternative explanation for the perfect conservation of sequences between pH8 and pH13 would be the existence of tandemly duplicated *H-2K* genes, although this seems not to be supported by observations from different laboratories. Only one gene among the 30–36 class I loci that have been cloned has been found to encode the *H-2K* antigen in transfection studies^{14,15}. Analysis of overlapping cosmid clones spanning 25 kilobases upstream and 50 kilobases downstream from the *H-2K* locus has revealed only one related sequence which has only 75% homology to the *H-2K* gene and which apparently does

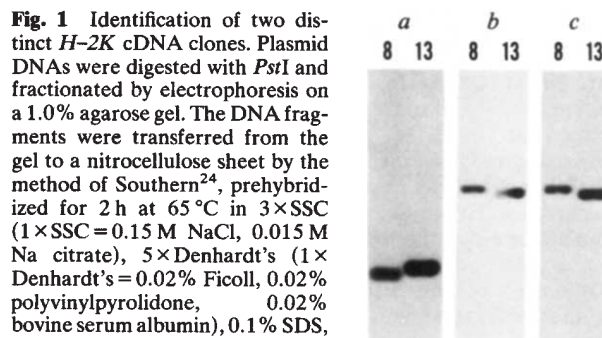
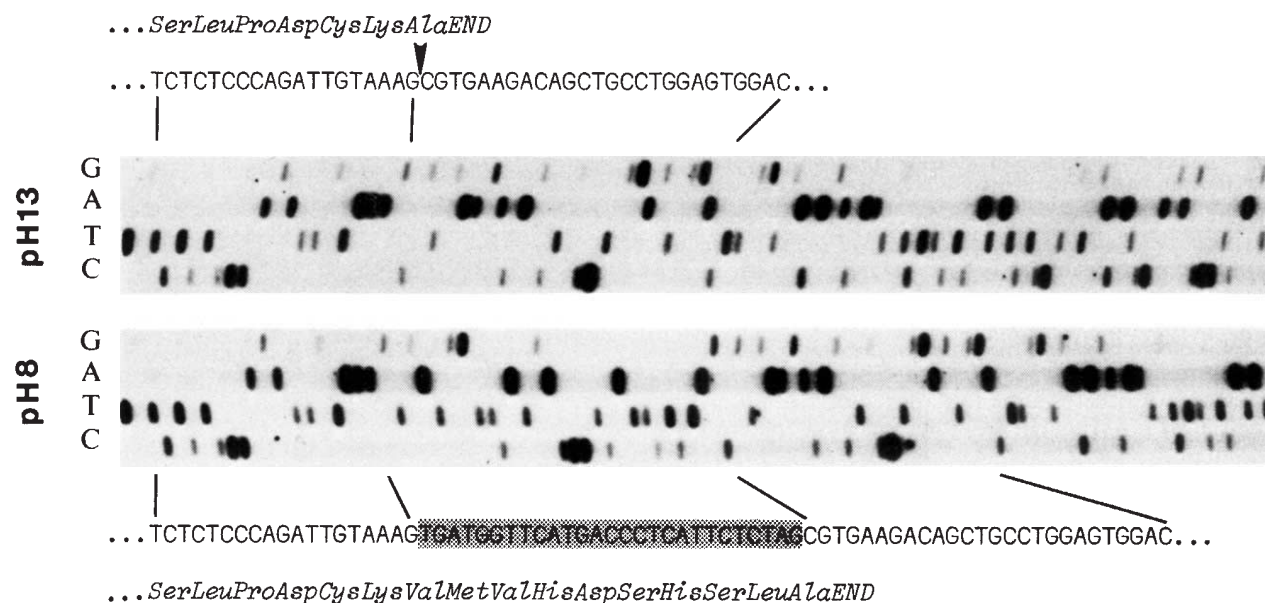


Fig. 1 Identification of two distinct *H-2K* cDNA clones. Plasmid DNAs were digested with *Pst*I and fractionated by electrophoresis on a 1.0% agarose gel. The DNA fragments were transferred from the gel to a nitrocellulose sheet by the method of Southern²⁴, prehybridized for 2 h at 65 °C in 3×SSC (1×SSC=0.15 M NaCl, 0.015 M Na citrate), 5×Denhardt's (1×Denhardt's=0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% SDS, and annealed for 16 h with one of several ³²P-labelled DNA probes. **a**, A ~200-bp *Pst*I fragment, derived from the extreme 3' noncoding region of the full-length *H-2K* cDNA clone pH22, was ³²P-labelled by nick-translation²⁵ and used as a hybridization probe in 3×SSC, 5×Denhardt's and 0.1% SDS at 65 °C. **b**, A ~575-bp *Pst*I fragment, derived from the full-length cDNA clone pH22 and extending from amino acid 237 to a site 239 bp downstream from the termination codon, was ³²P-labelled and used for **a**. **c**, A synthetic octadecadeoxyribonucleotide, corresponding to amino acids 295–300 of the *H-2K* gene was ³²P-labelled using [γ -³²P]ATP and polynucleotide kinase²⁶, and used as a hybridization probe in 6×SSC and 5×Denhardt's at 55 °C. Following hybridization, the nitrocellulose sheets were washed extensively, dried and exposed to Kodak XAR-5 films.

a SEQUENCE OF pH8 AND pH13

240 GGGGATGGAACCTTCCAGAAGTGGGCACTGTGGTGGTGCCTCTTGGGAAGGAGCAGTATTACATGCCATGTGTACCATCAGGGGCTGCCTAAGCCCTCCACCTGAGATGGAGCCTCCATCCGCT
 GlyAspGlyThrPheGlnLysTrpAlaSerValValValProLeuGlyLysGluGlnTyrTyrThrCysHisValTyrHisGlnGlyLeuProLysProLeuThrLeuArgTrpGluProProSerAla
 250 260 270 280
 GTCTCCAACACGGTAATCATTGCTGTTCTGGTTGCTTGGAGCTGCAATAGTCACTGGAGCTGTGGTGGCTTTTGTGATG []ATGAGGAGGAGAAACAGGTGGAAAGGAGGGGACTATGCTCTGGCT
 ValSerAsnThrValIleIleAlaValLeuValValLeuGlyAlaAlaIleValThrGlyAlaValValAlaPheValMet []MetArgArgArgAsnThrGlyGlyLysGlyGlyAspTyrAlaLeuAla
 290 300 310 320
 CCAGGCTCCCAGACCTCTGATCTGTCTCTCCAGATTGTAAAGTGAATGGTTTCATGACCCCTCATTCTCTAGCGTGAAGACAGCTGCCTGGAGTGGACTTGGTACAGACAATGCTTCTCATATCTCCTGTGA
 ProGlySerGlnThrSerAspLeuSerLeuProAspCysLysValMetValHisAspProHisSerLeuAla
 330 340
 CATCCAGAGCCCTCAGTTCTCTTTAGTCAAGTGTGTGATGTTCCCTGTGAGCCTATGGACTCAATGTGAAGAACTGTGGAGCCAGTCCACCCCTCCACACAGACCTGTCCCTGCACTGCTGTCTTCC
 CTCCACAGCCAACCTTGCTGGTTACAGCAAACTGAGGGACATCTGCAGCGTGTGAGCTCCATGCTACCTGACCTGCAACTCCTCACTTCCACTGAGAATAATAATTGAATGTAACCTTGATTGTT
 ATCATCTTGACCTAGGGCTGATTTCTGTTAATTTCATGGATTGAGAATGCTTAGAGGTTTTGTTTGTGTTGATTGATTGTTTTTTGAAGAAATAAGCATAGATGAATAAACTTCCAA

b**c** CYTOPLASMIC DOMAINS

CLONE	HAPLOTYPE	SEQUENCE
		Exon 6 (I1 Domain) Exon 7 (I2 Domain) Exon 8 (I3 Domain)
λK ^b	b	GTGGAAAGAGGGGACTATGCTCTGGCTCCAGGTTA...CTAGGCTCCCAGACCTCTGATCTGTCTCTCCAGATTGTAAAGGTGA...ACAGTGTGGTTTCATGACCCCTCATTCTCTAGCGTGA
pH8	q	lyGlyLysGlyGlyAspTyrAlaLeuAlaProG lySerGlnThrSerAspLeuSerLeuProAspCysLysV alMetValHisAspProHisSerLeuAlaEND
pH13	q	lyGlyLysGlyGlyAspTyrAlaLeuAlaProG lySerGlnThrSerAspLeuSerLeuProAspCysLysA laEND
λL ^d	d	-----GTTA...CTAG-----G-----AA-----G-----GTGA...ACAG-----T-----
pH12	q	lyGlyLysGlyGlyAspTyrAlaLeuAlaProG lySerGlnSerSerGluMetSerLeuArgAspCysLysA laEND

Fig. 2 Comparison of the nucleotide sequence of clones pH8 and pH13. **a**, *Pst*I fragments were subcloned into the multi-site M13mp8 vector²⁷ and sequenced by the primer extension procedure in the presence of 2',3'-dideoxynucleoside triphosphates²⁸. The sequences of two consecutive *Pst*I fragments, spanning ~780 nucleotides, are presented. The shaded region represents the sequence that is in pH8 but missing from pH13. The translated amino acid sequence is numbered according to the published *H-2K^b* (ref. 2). The termination codon and two consecutive polyadenylation signals are underlined. **b**, Autoradiogram of the sequencing gel indicating the only region of disparity between pH8 and pH13, an extra 27 nucleotides in the former. **c**, Alignment of cDNA sequences with the appropriate class I genomic sequence. Two *H-2K^b* cDNA sequences (pH8 and pH13) are compared with the *H-2K^b* genomic sequence⁹, and a *H-2K^b* cDNA sequence (pH12) is compared with the *H-2L^d* genomic sequence⁶. Only those sequences corresponding to the three exons encoding the three cytoplasmic domains are indicated. The intron between exons 6 and 7 is 173 nucleotides in length and the intron between exons 7 and 8 is 112 nucleotides long. Also indicated are the splice junctions and the translated amino acid sequence for each of the cDNA clones. The numbers represent positions of the amino acid residues².

not encode a detectable class I antigen^{9,14}. Furthermore, Southern blot analyses of total cell DNA from mice of different *H-2* haplotypes, using the *H-2K*-specific noncoding probe, revealed in each case a single hybridizing component (our unpublished results), an observation consistent with the existence of only one *H-2K* gene in the haploid genome. These observations strongly suggest that the two cDNA clones were derived from the same *H-2K^a* gene.

In an attempt to define the mechanism that generated the deletion observed in pH13 relative to pH8, we compared these cDNA sequences with the *H-2K^b* genomic sequence⁹. Figure 2c shows the alignment of pH8 and pH13 sequences with the λK^b sequence in the three cytoplasmic exons which encode the I1, I2 and I3 domains of the *H-2K* antigen. The deletion in pH13 is found precisely at the beginning of exon 8 and probably results from the use of an alternative splice acceptor site located 27 bases downstream from that used in pH8. Both splice acceptor sites conform to the consensus sequence that has been deduced by comparison of known sequences^{16,17}. At the translational level, both transcripts invoke the use of the same termination codon. The consequence of the use of alternative splice acceptor sites is the generation of two distinct polypeptide chains that would differ in length by nine amino acids, but would otherwise be perfectly identical.

We have so far analysed and sequenced five *H-2K*-specific cDNA clones isolated from the same liver cDNA library¹⁰. Of these, four clones (including pH8) utilize the upstream acceptor splice site and only one (pH13) utilizes the downstream acceptor splice site. While statistically it would be difficult to draw conclusions on the relative frequency of usage of the two acceptor sites, it seems reasonable to suggest that the upstream site is preferred. Consistent with this suggestion, three other *H-2K* cDNA clones, pH2^d-4 (ref. 18), pH2^d-33 (ref. 19) and pH202 (ref. 20), isolated by others from mice of the *d* or *b* haplotypes, all appear to use the upstream splice acceptor site like pH8.

The reason for the preferred use of the upstream acceptor site by transcripts of the *H-2K* gene is unclear. Interestingly, examination of the *H-2L^d* genomic sequence^{6,7} has also revealed the existence of potential alternative splice acceptor sites for the joining of exon 7 to exon 8. In our analysis and sequencing of four *H-2L* cDNA clones (including pH12), the downstream acceptor seemed to be used in all cases (Fig. 2c). Consistent with our finding, two other *H-2L* cDNA clones, pH2^d-3 (ref. 16) and pH203 (ref. 21), isolated from mice of the *d* and *b* haplotype, respectively, also used the downstream splice acceptor site like pH12. Because of the relatively small numbers of clones analysed, it is not clear if the upstream site can also be used in transcripts from the *H-2L* gene.

The use of the upstream splice acceptor site by the *H-2K* gene and the downstream site by the *H-2L* gene has previously been taken to suggest that differential use of splice sites exists between the two genes^{6-9,20}. Our isolation of distinct *H-2K* cDNA clones using either of the two acceptor sites strongly suggests the use of alternative splice sites in RNA transcripts expressed from the same class I gene.

While the intracellular region of the molecule has always been assumed to be important for interactions with cytoplasmic components³, and has been dissociated from the role of antigen presentation attributed to the extracellular domain, there has been no compelling reason for these assumptions. In fact, the only situation in which a biochemically stable complex involving class I antigens and a viral antigen was clearly demonstrated not to involve an interaction at the cell surface²². The suggestion from those studies was that the cytoplasmic domain may, in this case, be responsible for complex formation.

More recent studies using *in vitro* mutagenesis have demonstrated that while the cytoplasmic portion of the class I antigen may not be required for the presentation of the LCM virus antigen²³, this region of the molecule seems indispensable for interaction with the influenza virus antigen (J. G. Seidman, personal communication). The differences observed may depend on whether the viral determinants for recognition are located

on the cell surface, within the membrane matrix, or in the cytoplasmic aspect of the cell.

If, in fact, the cytoplasmic domain is required for such interactions, diversity in sequences in this part of the molecule would be a prerequisite. Mechanisms involving alternative RNA splicing could contribute to such diversity. Alternatively, differences in the cytoplasmic portion may suggest cell-specific roles for the class I antigens.

With an assay involving primer extension on RNA templates, we have confirmed the use of the two *H-2K* splice acceptor sites using total poly(A)⁺ RNA from mouse liver (data not shown). This direct assay should be valuable for confirming the use of alternative splice acceptor sites in heterologous cells transfected with a single *H-2K* gene and for estimating the relative use of alternative splice sites in different cell types.

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mRNA transcripts related to full-length endogenous retroviral DNA in human cells

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Mammalian cells contain multiple copies of endogenous type C retroviral DNA sequences¹⁻⁴. Among these sequences are complete, potentially infectious proviruses⁵⁻⁷, proviral DNA that is expressed only in the form of viral antigens⁸⁻¹⁰, retroviral segments that may contribute portions of envelope (*env*) genes during the generation of recombinant polytropic viruses^{11,12}, and many subgenomic viral DNA segments¹² that may not be expressed at all. We have previously reported the identification and molecular cloning of type C retroviral sequences from human DNA¹³ and have shown that the partial nucleotide and deduced amino acid sequences of one of the clones obtained

(λ 51) are homologous to Moloney MuLV (MoMuLV) in the *gag* and *pol* regions¹⁴. The λ 51 clone as well as several others isolated from a human DNA library contained approximately 4.3 kilobases (kb) of retroviral sequences, were deleted in the *env* region¹⁴, and were flanked by tandem repeats unlike the long terminal repeats (LTRs) typically found in proviral DNAs (P.E.S., in preparation). We describe here the characterization of a full-length human retroviral clone (λ 4-1) containing LTR elements as well as a putative *env* region. DNA-RNA hybridization experiments reveal that human cells contain species of poly(A)⁺ RNA that anneal to segments of the full-length retroviral DNA clone.

During the characterization of more than 30 endogenous human retroviral DNA clones, we noted that some (~20%), in addition to having extensive polynucleotide sequence homology with the truncated λ 51 prototype, contained several common restriction enzyme sites that were located 3' to MoMuLV-related *pol* gene sequences. The restriction maps of four of these clones (λ 4-1, λ 72-2, λ 97-1 and λ 12-2) are presented in Fig. 1 and aligned with both MoMuLV (panel *b*) and λ 51 DNA. DNA sequencing studies of the λ 51 class of retroviral DNAs have indicated a region of homology with MoMuLV extending 1.3–5.5 kilobases (kb) on the scale used in Fig. 1. λ 51 DNA and related human retroviral DNA segments contain no MoMuLV-related sequences 5' to base pair (bp) 1,350 nor *pol* sequences 3' to bp 5,471 (ref. 14 and P.E.S., in preparation). DNA sequence analysis also demonstrated that λ 51 DNA lacked an LTR element but instead contained a reiterated 72–75-bp segment that immediately flanked MuLV-related sequences (P.E.S., in preparation).

As shown in Fig. 1*a*, the second class of endogenous human retroviral sequences shares numerous restriction sites with the λ 51 prototype in the *gag* and *pol* regions (as indicated by the dotted symbols in Fig. 1) and, in addition, also contain unique restriction enzyme sites such as *Hind*III sites at 5.5 and 7.3 kb and a *Bam*HI site at 6.3 kb that are located within a region that would correspond to the *env* gene of MoMuLV. While three of the clones in this second class terminate in the *gag* or *pol* regions, one (λ 4-1) extends a sufficient distance in the 5' direction to encompass a potentially full-length proviral DNA. This clone retains numerous restriction enzyme sites characteristic of MuLV-related *gag* and *pol* regions and, in addition, contains paired *Acc*I and *Sac*I sites at 0.4 and 0.5 kb and at 8.8 and 8.9 kb. The *Acc*I/*Sac*I sites at 8.8 and 8.9 kb are also present in the other members of this class (Fig. 1). This spacing (a separation of ~8.4 kb) of the *Acc*I/*Sac*I sites in the λ 4-1 clone would be compatible with their location within LTR elements. Heteroduplex analyses as well as cross-hybridization studies (data not shown) have demonstrated that a 550-bp *Taq*I fragment encompassing the 5' *Acc*I and *Sac*I sites of λ 4-1 (and located between 0 and 550 bp, as shown in Fig. 1*b*) hybridized to restriction fragments containing the 3' *Acc*I/*Sac*I sites of λ 4-1 as well as to comparable regions of λ 72-2, λ 97-1 and λ 12-2 DNAs. No annealing of the cross-hybridizing *Taq*I fragment to clones of the λ 51 class was observed. DNA sequence analysis (P.E.S., manuscript in preparation) indicates that the *Acc*I and *Sac*I sites lie within nearly identical 495–499-bp segments that are located at 0–0.5 kb and 8.4–8.9 kb (Fig. 1) and contain several regulatory signals characteristic of retroviral LTRs^{15,16}: a polyadenylation site, a putative TATA box, a possible tRNA primer-binding site adjacent to the 5' LTR, and a polypurine tract adjacent to the 3' LTR. Although the putative *env* region of the second class of cloned human retroviral DNAs failed to hybridize to the *env* regions of several primate and murine type C retroviruses (A. Rabson, unpublished observations), nucleotide sequencing of this region (P.E.S., in preparation) has revealed a potential *env* mRNA splice-acceptor site in λ 4-1 DNA that coincides with the one present in MoMuLV (at 5.9 kb)¹⁷ and which is followed by a long open reading frame presumably coding for an *env* gene product. Thus, λ 4-1 exhibits features typical of an 8.8-kb type C endogenous provirus including LTR elements *gag*, *pol* and putative *env* sequences.

The presence of multiple copies of full-length endogenous retroviral sequences in the human genome raises the possibility that some are transcribed into RNA. We were particularly interested in studying tissues in which expression of retroviral-related proteins had been previously described in both human and non-human systems. These tissues included placentas, in which type C particles have been repeatedly observed in human and non-human primates^{18,19}, and lymphoid tissue and secretory epithelium, sites of endogenous gp70 protein production in the mouse^{20–22}. Northern blot analyses indicated that two different human placentas contain substantial amounts of a 3.0-kb RNA species that hybridizes to LTR (Fig. 2*a*, lanes 2, 3) and *env* (Fig. 2*a*, lanes 7, 8) DNA probes. A second, 1.7-kb *env*-reactive RNA was also present in both placentas which, in one (lane 3, Fig. 2*a*) of the two preparations, also annealed to the LTR probe. The 1.7-kb RNA was also detected in a human colon carcinoma line (Fig. 2*a*, lanes 5, 10) and, to a lesser extent, in liver and spleen preparations (Fig. 2*a*, lanes 4, 6, 9). Variable amounts of 3.6- and 2.2-kb LTR-reactive poly(A)⁺ RNAs were detected in the human cells examined, being particularly prominent in colon carcinoma cells (Fig. 2*a*, lane 5). The relationship of this RNA and a co-migrating species present in the two placentas which hybridizes to the *env* probe is currently under study. No hybridization of poly(A)⁺ RNA present in liver, placenta, spleen or colon carcinoma cells to the *gag* or *pol* probes was detected (data not shown).

For purposes of comparison, Fig. 2*b* shows the sizes of MuLV *env*-reactive RNAs present in virus-producing AKR mouse thymoma cells. In addition to the 8.8-kb full-length viral RNA, these cells contain a 3.0-kb (21S) spliced MuLV LTR-*env* RNA species that co-migrates with the 3.0-kb LTR and *env*-reactive RNAs detected in human placenta and colon carcinoma cells.

Because of the association of endogenous retroviruses with the development of leukaemia in the mouse⁶ and the association of human T-cell leukaemia virus (HTLV) with this disease in man^{23,24}, we have begun to study the expression of human retroviral sequences in a variety of lymphoid malignancies. Using dot-blot hybridization (data not shown), no RNA hybridizing to our retroviral probes was detected in three malignant B-cell lines (BJAB, Raji, Nalm I); however, a T-cell acute lymphocytic leukaemia line, 8402 (which does not contain HTLV sequences)²⁵, exhibited strong reactivity and was, therefore, studied by Northern blot analysis. A 6.8-kb RNA species reactive with the *gag*, *pol* and *env* probes (Fig. 2*c*, lanes 2–4) was detected. This RNA failed to hybridize to the LTR probe (lane 1), implying either that it does not contain LTR sequences or is associated with an LTR that differs from the element present in the λ 4-1 class. The T cells also contain a 4.8-kb RNA that hybridizes to all four human retroviral probes. Whether these retroviral RNAs contribute to the malignant phenotype of the leukaemia cell line must await study of additional T-cell samples, both normal and malignant. Nonetheless, the presence of *pol*-reactive mRNA in these cells is of interest as transcription of endogenous *pol* genes could provide the source of reverse transcriptase used in the generation of pseudogenes²⁶.

Thus, human cells contain RNA species transcribed from different regions of potentially full-length endogenous retroviral sequences. These transcripts vary in size, content and quantity from tissue to tissue in a manner analogous to that seen in the mouse²⁷. One interesting feature of the mouse system has been the consistent association of endogenous gp70 RNA and protein expression with cells of the reproductive tract, particularly epididymis and gravid uterus²⁰. The fact that term human placentas also contain *env*-related mRNAs further supports the possibility that endogenous *env* gene products have a functional role in these tissues. As gp70 is important in virus-cell interactions²⁸, its presence on the surface of normal cells could inhibit the adsorption of related infectious retroviruses. This could be of particular importance in trophoblastic tissue, since it would represent a barrier to transplacental infection of developing embryos, thus preventing potential mutagenic^{29,30} or adverse teratogenic effects. Furthermore, the presence of retroviral *env*

Fig. 1 Identification of a cloned full-length human retroviral DNA. **a**, Restriction endonuclease maps of five human retroviral DNA segments isolated from a human gene library³¹ as previously described^{13,14}. The cloned retroviral DNAs were aligned with one another on the basis of common restriction sites and with MoMuLV proviral DNA by reciprocal DNA-DNA hybridization experiments¹³ and nucleotide sequence determinations encompassing the shaded *gag* and *pol* regions shown diagrammatically in **b**¹⁴. Restriction enzyme sites common to the cloned DNAs are indicated by letters shown with a dot above or below. A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; S, *Sac*I. **b**, Restriction enzyme sites defining subgenomic DNA probes isolated from a full-length human retroviral clone (λ 4-1). B, *Bam*HI; R, *Eco*RI; H, *Hind*III; P, *Pst*I; T, *Taq*I.

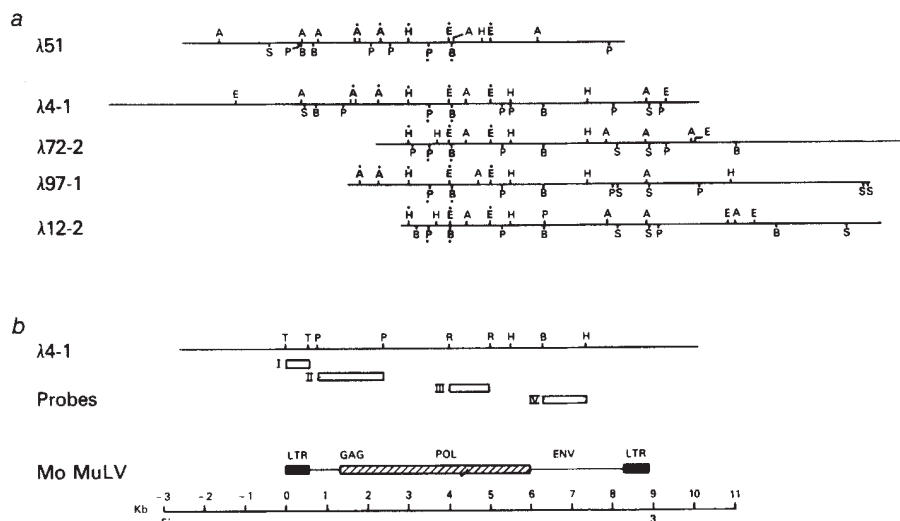
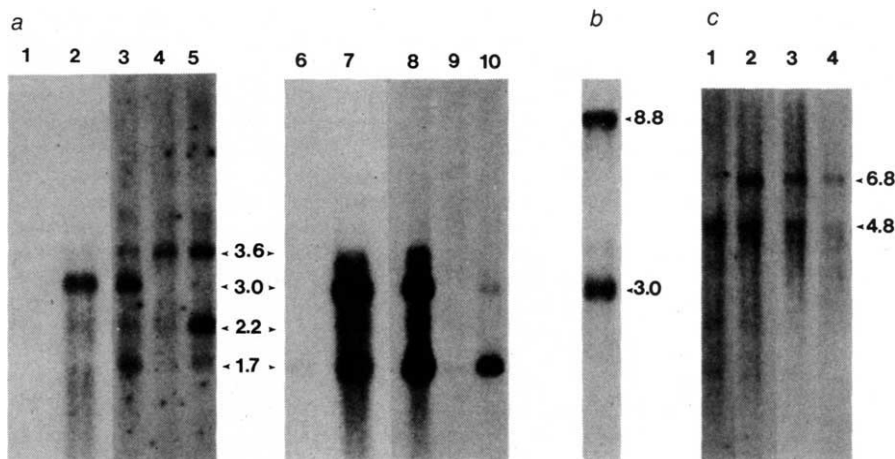


Fig. 2 Northern hybridization analysis of retroviral-related RNAs. **a**, Poly(A)⁺ RNAs were prepared from human liver (lanes 1, 6), two full-term human placentas (lanes 2, 7 and 3, 8), human spleen (lanes 4, 9) and a human colon carcinoma line, SW1116³² (lanes 5, 10). RNAs were electrophoresed through denaturing gels, transferred to nitrocellulose filters and hybridized to ³²P-labelled DNA probes derived from λ 4-1, as described below. The radiolabelled DNA probes used included an LTR segment (probe I, Fig. 1b), lanes 1-5, and an *env* segment (probe IV, Fig. 1b), lanes 6-10. **b**, Total cellular RNA prepared from an AKR mouse thymoma was hybridized to a radiolabelled envelope segment from AKR ecotopic MuLV³³ following electrophoresis and transfer to a nitrocellulose filter. **c**, Poly(A)⁺ RNA samples extracted from a T-cell acute lymphocytic leukaemia line, 8402³⁴, were transferred to nitrocellulose membranes following electrophoresis. Nitrocellulose filters were hybridized to radiolabelled DNA segments of λ 4-1; lane 1, LTR probe; lane 2, *gag* probe (probe II, Fig. 1b); lane 3, *pol* probe (probe III, Fig. 1b); and lane 4, *env* probe.



Methods: RNAs were extracted by a modification of the guanidine hydrochloride procedure³⁵ using 5 mM vanadyl ribonucleoside compounds³⁶ during the initial homogenization. Total cellular RNA was subjected to one cycle of chromatography on oligo(dT) cellulose³⁷. 5- μ g aliquots of poly(A)⁺ or total cellular RNA were heated at 65 °C for 10 min in a mixture containing 50% formamide, 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 5 mM NaOAc and 1 mM EDTA and electrophoresed through 1% agarose gels containing 2.2 M formaldehyde³⁸ for 14-16 h at 30 V in a buffer containing 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 5 mM NaOAc and 1 mM EDTA. Following electrophoresis, RNA was transferred to nitrocellulose membranes in 20 $\times$ SSC³⁹ (1 $\times$ SSC=0.15 M NaCl, 0.015 M Na-nitrate) and baked for 2 h at 80 °C. Membranes were prehybridized for 5 h at 45 °C in 50% formamide 5 $\times$ SSC, 0.1 M Tris (pH 7.5), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's=0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 300 μ g ml⁻¹ yeast RNA and 10% dextran sulphate⁴⁰. Nic-translated ³²P-labelled DNAs⁴¹, specific activity 4-10 $\times$ 10⁸ c.p.m. per μ g, were added to the prehybridization solution at a final concentration of 0.5 $\times$ 10⁶ c.p.m. ml⁻¹ and the filters were incubated for 16-24 h at 45 °C. Following hybridization, filters were washed at 50 °C in 2 $\times$ SSC, 0.1% SDS for 10 min and in 0.1 $\times$ SSC, 0.1% SDS for 30 min. The membranes were air-dried and exposed to X-ray film at -70 °C for 4-7 days (**a**, **c**) or 12 h (**b**). Sizes in kilobases were determined by comparison with 28S and 18S ribosomal RNA bands visualized by acridine orange⁴² staining of a marker lane excised from each gel before transfer.

gene products on the surface of normal cells might mediate important cell-to-cell interactions, particularly in an organ such as the placenta where cells with different histocompatibility antigens (fetal and maternal) come into intimate contact.

An interesting feature of the RNA analyses described here was the detection of poly(A)⁺ RNA in certain cells (such as the 3.6- and 2.2-kb species present in spleen and colon carcinoma cells) that hybridized to the LTR probe (Fig. 2b, lanes 4, 5) but not to any other labelled human retroviral DNA. Such RNAs could reflect the activity of a 3' LTR and the associated downstream transcription of an adjacent cellular gene. In this regard

we have recently obtained several clones from a human DNA library which contain an LTR-reactive segment but no associated retroviral sequences (M.A.M., in preparation). Initiation of RNA synthesis within a few of these widely dispersed and 'unaffiliated' LTR elements could lead to the expression of neighbouring cellular genes and generate some of the discrete transcripts visualized in Fig. 2a.

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Actively transcribed genes are associated with the nuclear matrix

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In the chicken oviduct, it has been well documented that steroid hormones stimulate the transcription of specific genes such as the ovalbumin gene¹⁻⁷. In addition to the presence of specific hormone receptors in the tissue, gene expression seems to require that target genes exist in large DNase I sensitive chromosomal domains^{2,8}. This structure appears necessary but not sufficient for transcriptional activation. In search of still other levels of control, we have investigated the interactions of genes with the nuclear matrix, a structure which has been implicated in DNA synthesis, transcription and RNA processing⁹⁻¹². Here we have isolated nuclear matrix and used a nondegradative method to fractionate nuclear DNA based on its preferential association with the matrix. The preparation was digested with a restriction enzyme and both matrix-bound and released DNAs were recovered. We found that only actively expressed genes were associated with the matrix. Furthermore, within a 100-kilobase (kb) DNase I sensitive chromosomal domain, only the transcribed regions were associated with the matrix. This association was shown to be reversible when hormone was withdrawn. Our results suggest that the nuclear matrix is the site of nuclear transcription and may represent another potential level of control for regulation of gene expression in the eukaryotic cell.

Table 1 DNA associations with the nuclear matrix

Tissue	Genes	
	Enriched in matrix	Depleted in matrix
Hormonally-stimulated oviduct	Ovalbumin	Globin
	X	Domain 2.7 (5')
	Y	Domain 6.8 (5')
	Ovomucoid	Domain 11.8 (3')
Hormonally withdrawn oviduct	β -Actin	Vitellogenin
		Ovalbumin
Liver (hen or rooster)		Ovalbumin

DNA attached to a protein matrix was isolated from hen oviducts and digested with the restriction enzyme *EcoRI*. The released (85% of total) and bound (15% of total) fractions were separated quantitatively and analysed by gel electrophoresis, blotted to nitrocellulose by the method of Southern¹³ and hybridized to a probe specific for both the chicken ovalbumin gene and a portion of the chicken β -globin gene. The results are shown in Fig. 1a. The pattern of hybridization in the released fraction matched that of total nuclear DNA but the bound fraction was found to be substantially enriched in ovalbumin DNA sequences. In contrast, globin sequences were depleted in the matrix-bound fraction. As the ovalbumin gene is transcribed in the oviduct and the globin gene is not transcribed, this suggests that transcriptional activity of a gene may be correlated with its association with the nuclear matrix. To examine this correlation further, we looked at the association of various regions of the entire ovalbumin gene. Figure 1b shows the results. Three probes were prepared which were specific for various regions in and surrounding the ovalbumin gene as seen in Fig. 1. All showed an enrichment in the matrix-bound fraction. Thus, the entire ovalbumin transcriptional unit is associated with the oviduct nuclear matrix.

To determine whether this was a general phenomenon of transcribed genes, the association of other genes in the ovalbumin multigene family was investigated. These results are shown in Fig. 1c. The X and Y genes are homologous to the ovalbumin gene but are transcribed at lower rates in hormonally stimulated chickens¹⁴. Despite these differences in transcriptional levels, both X and Y genes were found to be associated with the stimulated oviduct matrix, similar to the ovalbumin gene.

In our laboratory we have defined a 100-kb DNase I sensitive domain containing the ovalbumin multigene family⁸. This structurally distinct chromatin domain has been identified by studies of the relative sensitivity of various portions of the genome to DNase I in intact nuclei. It was of particular interest to see if the transition regions of this domain would act as attachment points of a looped structure to the protein matrix. Figure 2 shows that association of various regions of the domain is confined only to those regions containing transcriptionally active genes. Neither the DNase I sensitive but nontranscribed flanking regions nor the DNase I resistant regions at both ends of the domain are associated with the matrix. Therefore, there is no general structural correlation between the DNase I sensitive chromosomal domain and the nuclear matrix. In contrast, a strong correlation exists between gene transcription and attachment to the matrix.

The oviduct system provides an excellent opportunity for altering the transcriptional state of a particular hormonally responsive gene. Immature chicks can be stimulated with hormone, in which condition the ovalbumin gene is transcribed; hormone can be removed and transcription is discontinued⁷. Matrix was isolated from such hormonally withdrawn chick oviducts and the analysis of the association of the ovalbumin gene is shown in Fig. 3. In the absence of transcription in the hormonally withdrawn oviduct, the ovalbumin gene was no longer associated with the oviduct matrix. Thus, withdrawal of

Fig. 1 *a*, A nitrocellulose filter was hybridized with a 32 P-labelled probe containing sequences within the 12-kb *Eco*RI fragment 5' of the ovalbumin gene (OV) and a 32 P-labelled probe within the 6.1-kb *Eco*RI fragment containing the chicken β -globin gene (GL). The genes of interest are shown schematically in a 5' to 3' direction below the autoradiographs. The *Eco*RI sites are designated by arrows and the numbers refer to the kilobases between particular *Eco*RI sites. *b*, Nitrocellulose filters were hybridized with three 32 P-labelled probes. One probe was a 9.2-kb *Eco*RI fragment from the 3' end of the ovalbumin gene. The other two probes were the 2.4-kb and 1.8-kb *Eco*RI fragments within the ovalbumin structural gene. *c*, The left three lanes were hybridized with ovalbumin and globin gene probes similar to those used in *a*. Another probe containing a portion of the 3.4 kb *Eco*RI fragment at the 3' end of the X gene was also added. The other lanes were hybridized with a labelled 1.8-kb *Eco*RI fragment within the ovalbumin gene and a segment of the 7.2-kb *Eco*RI fragment at the 5' end of the Y gene.

Methods: Nuclei were isolated and purified from chicken oviduct or liver tissue as described previously⁸. All procedures were performed at 4 °C unless otherwise noted and all liver matrix preparations were performed in the presence of 1 mM phenylmethylsulphonyl fluoride. The nuclei were lysed in a buffer containing 2 M NaCl, 0.5% Triton, 10 mM Tris pH 8.0 and 10 mM EDTA, and rocked gently for 15 min. The loop containing structures were then purified from the soluble proteins by sedimentation through a step gradient of 15% sucrose with 2 M NaCl, 10 mM EDTA, 10 mM Tris pH 8.0 layered over a cushion of 30% sucrose in a similar buffer. This was centrifuged at 8,000 r.p.m. for 120 min. The material at the interface of the 30% sucrose cushion was collected, washed extensively with *Eco*RI digestion buffer and incubated at a concentration of 0.1 mg ml⁻¹ with 5 U or enzyme per μ g of DNA. After 30 min at 37 °C the mixture was centrifuged for 30 min at 10,000 r.p.m. and the supernatant fraction was immediately removed. A sample from both fractions was deproteinized as described previously⁸, treated with DNase-free RNase A at a concentration of 100 μ g ml⁻¹ for 30 min at 37 °C and then treated with proteinase K at a concentration of 10 μ g ml⁻¹ for an additional 30 min at 37 °C. The samples were passed through a Sephadex G-50 column and the DNA was ethanol precipitated before recutting to completion with *Eco*RI. 10 μ g of each sample was electrophoresed in a 1% agarose gel. The DNA was transferred to nitrocellulose paper by the method of Southern¹³, the paper was baked for 6 h in a vacuum at 68 °C, prehybridized with 6 $\times$ SSC and 2 $\times$ Denhardt's reagent overnight at 68 °C and then hybridized overnight at 68 °C in 6 $\times$ SSC, 2 $\times$ Denhardt's reagent, 1 mM EDTA, 0.5% SDS and 20 $\times$ 10⁶ c.p.m. of a specific 32 P-labelled plasmid probe⁸. The filter was then washed at 68 °C for 2 h with 1 $\times$ SSC and 0.5% SDS and rinsed with 1 $\times$ SSC. Kodak XRP-1 film was exposed to the filter with an intensifying screen overnight at -70 °C.

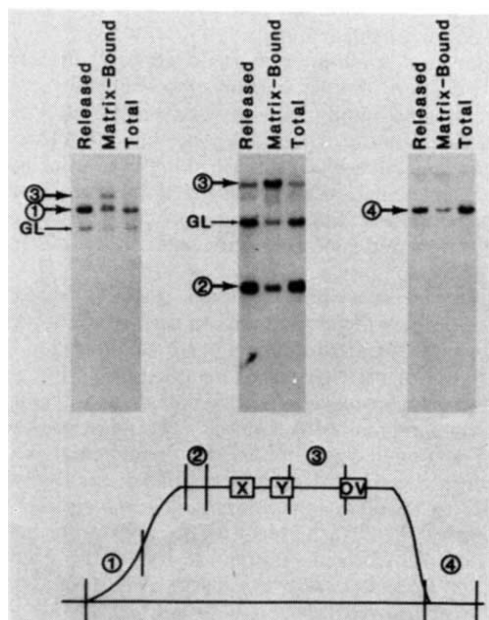
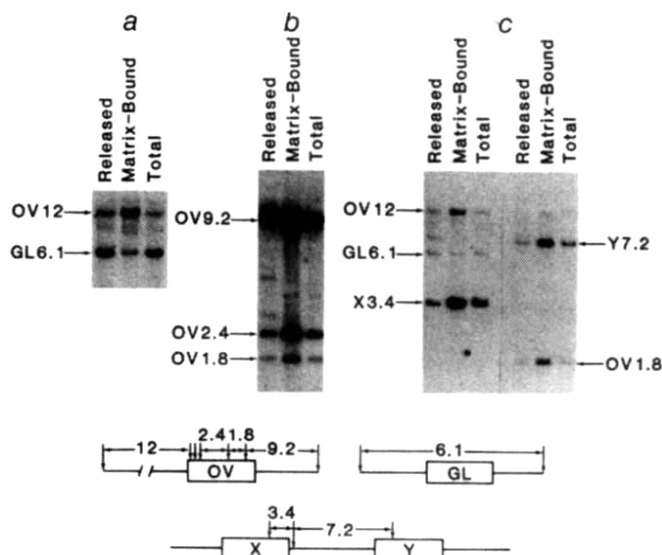


Fig. 2 Samples were isolated and treated as for Fig. 1. Nitrocellulose filters were hybridized with probes containing sequences within the ovalbumin 100-kb DNase I sensitive domain. The diagram below the autoradiographs shows the location of the probes within the *Eco*RI fragments (vertical lines) of the 100-kb domain. The vertical distance from the horizontal line represents the relative sensitivity to DNase I of that region (for example, 2 is sensitive and 4 is resistant).

hormone causes both the release of the gene sequences from the matrix structure and the termination of their transcription. Table 1 summarizes our data. In addition to the results presented here, we have examined other hormonally regulated genes

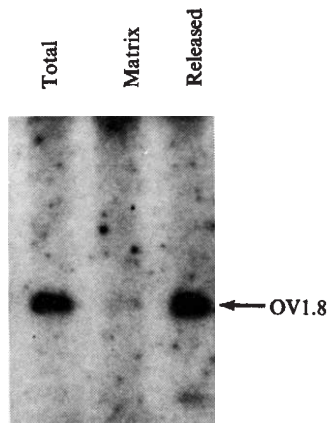


Fig. 3 Samples were isolated and treated as for Fig. 1. DNAs released from or associated with the nuclear matrix of withdrawn oviduct were probed with a sequence, OV 1.8, within the ovalbumin coding region.

to determine whether attachment was a general property of such hormonally responsive genes. The gene for ovomucoid is under hormonal regulation, similar to ovalbumin in the chicken oviduct but is not a member of the ovalbumin gene family. This gene was also found associated with the oviduct nuclear matrix. Vitellogenin, although hormonally regulated but expressed only in the liver, was not found associated with the oviduct matrix. We have also found that in the liver, a tissue where ovalbumin is not transcribed, the gene is not associated with the matrix. Finally, we have examined another non-hormonally stimulated gene, the β -actin gene. We found this gene to be associated with the oviduct matrix. This result is consistent with the finding that the chicken β -actin gene is expressed in the oviduct. Therefore, all the transcribed genes studied to date, whether hor-

monally regulated or not, have been associated with the nuclear matrix structure. In contrast, we have shown that nontranscribed sequences are not bound to the matrix.

Thus, we have found that of 11 genomic sequences examined, only actively expressed genes were quantitatively bound to the nuclear matrix. Nontranscribed sequences were localized in the unattached 'loop' structures and could be released by restriction enzyme treatment. Also, the association of the ovalbumin gene with the nuclear matrix was reversible when hormone was withdrawn and transcription ceased. Our composite results suggest that in addition to containment within DNase I sensitive chromosomal domains, expression of genes may require associ-

ation with the nuclear matrix. These results suggest that another structural level of control for gene expression may exist in the nucleus.

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Note added in proof: After this manuscript was submitted a similar report appeared, substantiating these results¹⁵.

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Sequence of cDNA encoding human insulin-like growth factor I precursor

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Somatomedins (SM) or insulin-like growth factors (IGF) constitute a heterogeneous group of peptides¹ with important growth-promoting effects *in vitro*^{2,3} as well as *in vivo*^{4,5}. Amino acid sequences have been determined for only two of them, IGF-I and IGF-II, which are highly homologous^{6,7}. IGF-I, which is identical with SM-C⁸, is composed of 70 amino acid residues and IGF-II contains 73 amino acids and may be identical with SM-A⁹. Other peptides with different charge properties but with similar SM-like or insulin-like behaviour in biological and receptor assays, have been described but have not yet been fully characterized¹. The liver is known to be a major site of production of these peptides¹⁰, but many other tissues—especially in the fetus¹¹—may synthesize them as well. We report here the nucleotide sequence of a human liver cDNA encoding the complete amino acid sequence of IGF-I. The IGF-I coding region is flanked by sequences encoding an amino-terminal peptide of at least 25 amino acid residues and a carboxyl-terminal peptide of 35 amino acids. This provides evidence that IGF-I is synthesized as a precursor protein and that formation of IGF-I from this precursor requires proteolytic processing at both ends.

For the isolation of IGF-I cDNA we used a cDNA library derived from human adult liver¹². This library was constructed by preparing double-stranded cDNA from poly(A)-containing mRNA and inserting it into the *Pst*I restriction site of the pBR322 derivative pKT218¹³, using the dG-dC tailing technique. Of the 230,000 tetracycline-resistant transformants of *Escherichia coli* MC 1061¹⁴ approximately 60,000 were screened with synthetic oligonucleotide probes. From the amino acid sequence of IGF-I⁶ the region containing amino acids 58-62 (Glu-Met-Tyr-Cys-Ala) was selected for the synthesis of the corresponding nucleotide sequence. A mixture of all eight possible tetradecamer oligonucleotides (5'-G-A-G-A-T-G-T-A-C-T-G-C-3', excluding the third base of the alanine codon), was chemically synthesized by using a solid phase phosphate triester method¹⁵. After 5'-

labelling using [γ -³²P]ATP and T₄ polynucleotide kinase¹⁶, the oligomer mixture was purified by electrophoresis on a 20% polyacrylamide gel. Subsequently, the ³²P-end-labelled tetradecamers were used to screen the cDNA library as described by Woods *et al.*¹². Twenty bacterial clones showing hybridization with the labelled oligonucleotide probe were selected and rescreened with the same oligonucleotide mixture. This resulted in the isolation of 5 clones showing unambiguous hybridization.

One of these clones contained a DNA insert of 777 nucleotides, which was subjected to nucleotide sequence analysis according to Maxam and Gilbert¹⁷. Both strands of the DNA were completely sequenced following the strategy depicted in Fig. 1. The nucleotide sequence of the cDNA strand homologous to the corresponding mRNA is shown in Fig. 2. This sequence contains a long open reading frame of 470 nucleotides, consisting of a region of 210 nucleotides corresponding to the complete amino acid sequence of IGF-I, flanked at the 5'-end by a region of 155 nucleotides and at the 3'-end by 105 nucleotides. This open reading frame is followed by a 3'-nontranslated region of 255 nucleotides and a poly(A) tract. The remainder of the sequence is accounted for by the poly(dC) and poly(dG) tails.

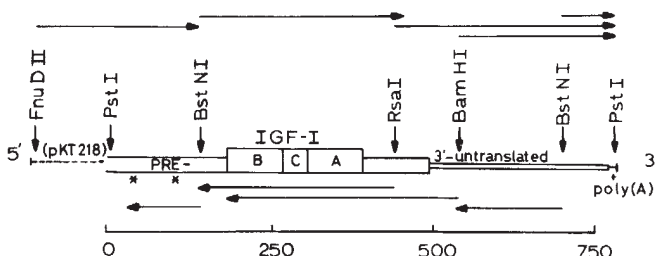
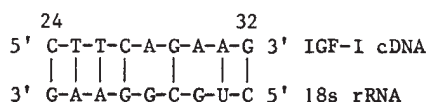


Fig. 1 Organization and sequencing strategy for the cDNA insert in pIGF-I. The regions coding for the B-, C- and A-chains of IGF-I (higher open boxes), as well as those coding for the N-terminal and the C-terminal peptide (lower open boxes) are schematically depicted. Of the restriction sites, only those used for sequencing are indicated. The *Fnu*DII site is located in pKT 218, 107 nucleotides from the *Pst*I site. Nucleotide sequence analysis of the two strands of the cloned cDNA insert was performed according to Maxam and Gilbert¹⁷, with slight modifications in order to determine sequences of fragments longer than 250 nucleotides²⁹. The horizontal arrows indicate the direction and extent of the sequence determinations. The box representing the coding region for the N-terminal peptide is left open to the 5'-side, indicating the uncertainty as to whether this cDNA contains the entire coding region. Asterisks below this box indicate the two ATG codons at which the translation could theoretically start. The calibrated horizontal bar underneath represents the number of nucleotides.

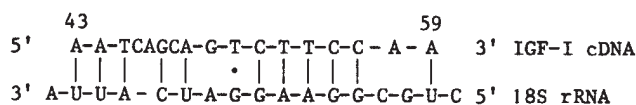
The nontranslated 3'-terminal region contains a variant polyadenylation signal, AATAAT, 20 nucleotides upstream from the poly(A) tract. This signal is identical to that found in the mRNA of a structurally related peptide, rat preprorelaxin¹⁸.

The first ATG codon in the open reading frame is found at nucleotide positions 35-37 (Met 48) which, if used for initiation of translation, would result in the synthesis of a precursor protein consisting of 153 amino acid residues. In this protein, the IGF-I amino acid sequence would be preceded by a peptide of 48 amino acids. In this respect it is noteworthy that to the 5'-side of ATG 35-37 (Met 48) a sequence of nine nucleotides is found, with a high degree of complementarity to the 3'-end of 18S ribosomal RNA¹⁹:



There is evidence that base-pairing between some eukaryotic mRNAs and the 3'-end of 18S rRNA might play a role in the initiation of eukaryotic translation^{20,21}.

Another ATG codon, located at nucleotide positions 104-106 (Met 25), is also preceded by a sequence which shows a similar degree of complementarity to the 3'-end of 18S rRNA:



Use of this ATG codon for initiation of translation would lead to the synthesis of a precursor protein consisting of 130 amino acid residues, including a peptide of 25 amino acids at the N-terminal end of the IGF-I sequence. The length of this peptide and the highly hydrophobic stretch of amino acids contained in it (Leu 17 to Phe 7) suggests a signal function²² and makes this codon an attractive site for the initiation of translation.

However, we cannot exclude the possibility that the actual 5'-end of the IGF-I mRNA is not present in this cDNA clone and that initiation of translation occurs upstream from the sequenced region. This would lead to the synthesis of an IGF-I precursor of more than 156 amino acid residues. Two recent observations are relevant in this context: (1) cell-free translation of rat IGF-II results in the formation of a 21,600 molecular weight protein (approximately 200 amino acids)²³; and (2) SM activity recovered from rat liver extracts has an even higher apparent molecular weight of approximately 30,000²⁴. Detailed analysis of intracellular mRNA and radiosequencing of the primary translation product will be necessary to resolve this issue.

The IGF-I precursor has an additional peptide of 35 amino acid residues at its carboxyl terminus. A C-terminal adjacent peptide has also been described for the precursor of human calcitonin²⁵. This peptide exerts biological activity²⁶. The amino acid sequence of both the N-terminal and the C-terminal peptides was subjected to a computer analysis in search of homologies with known proteins (search conducted by Dr W. Barker at the US National Biomedical Research Foundation, Georgetown University). No distinct homologies were found; this, however, does not preclude a biological function of these peptides.

Considerable homology in amino acid composition exists between IGF-I and IGF-II (62%), and also between these peptides and proinsulin⁶. This amounts to 48% (14/29) amino acid sequence homology between the B-chains of IGF-I and proinsulin; at the nucleotide level²⁷, the overall degree of homology is 53% (48/90), despite a considerable number of nucleotide changes in the codons for the homologous amino acids. For the A-chains, the figures are 52% (11/22) and 57% (36/63) respectively. Here, too, more than half of the codons for homologous amino acids show nucleotide changes in the third position. The N-terminal regions show only 32% (23/72) nucleotide sequence homology; interestingly, the second ATG codon in the IGF-I N-terminal sequence (nucleotide positions

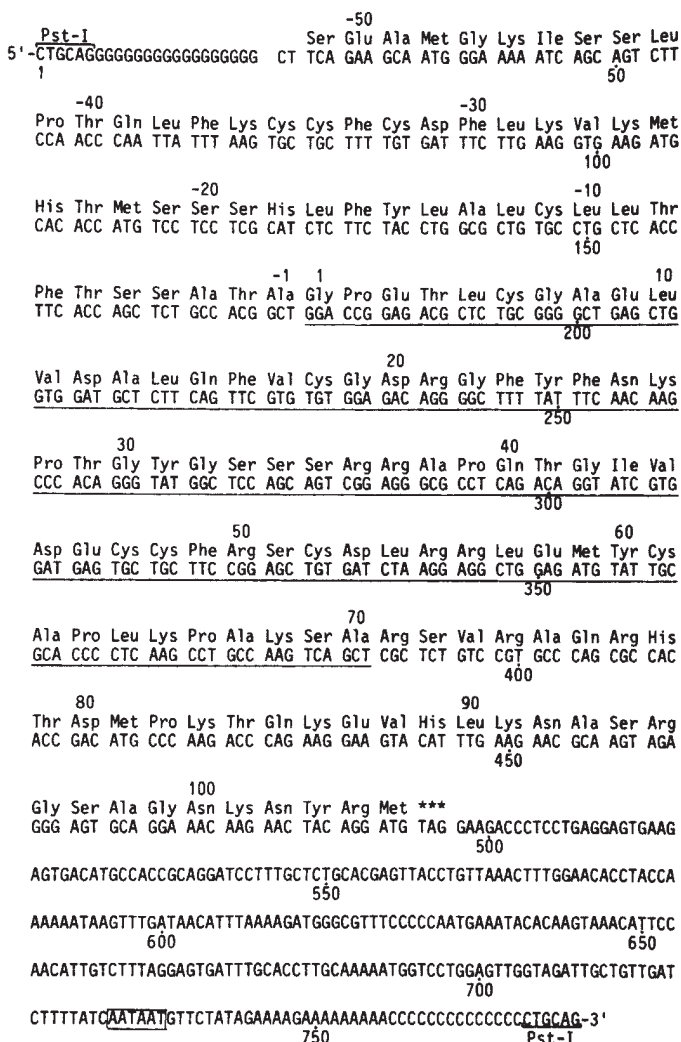


Fig. 2 Nucleotide sequence of the cDNA insert in pIGF-I. The DNA strand presented is homologous to IGF-I mRNA. Nucleotides are numbered sequentially from the *Pst*I restriction site at the 5'-end. The predicted amino acid sequence shown above is numbered sequentially from the amino terminus of the IGF-I molecule, through its 70 amino acid residues (underlined) and the 35 amino acids attached to its C-terminus. The amino acids preceding IGF-I are given negative numbers. The asterisks indicate the termination codon, TAG. The variant polyadenylation signal, AATAAT, located in the 3'-nontranslated region 20 nucleotides upstream from the poly(A) tract, is boxed.

104-106; Met 25) is found at the same distance, 5' to the first region of homology, as the initiation codon of preproinsulin²⁷.

Comparison of the nucleotide sequence of IGF-I with another structurally related peptide, relaxin²⁸, results in 31% (9/29) amino acid homology in the B-chains and 32% (28/89) nucleotide sequence homology. For the A-chains, these percentages are 35% (7/20) and 49% (29/60) respectively.

The finding that IGF-I is synthesized as a precursor protein raises the question where and how this precursor is processed. In this respect, it must be emphasized that the characterization of the large proteins with which SM activity is associated in native plasma, and of a number of the smaller peptide fractions with SM characteristics which are released after acidification, is still incomplete. One of these circulating forms could very well be pre-IGF-I, which is subsequently converted into IGF-I by proteolytic processing.

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Isolation and structural organization of the human preproenkephalin B gene

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The primary structure of porcine preproenkephalin B has been elucidated by cloning and sequencing cDNA¹: it contains neoendorphin^{2,3}, dynorphin^{4,5} and leuromorphin^{1,6,7} (containing rimorphin as its amino-terminus)^{8,9}. These opioid peptides, each having a leucine-enkephalin structure¹⁰, act on the κ -receptor^{7,11–14}. We have now cloned a human genomic DNA segment containing the preproenkephalin B gene. The structural organization of this gene resembles those of the genes encoding the other opioid peptide precursors, that is, preproenkephalin A¹⁵ and the corticotropin- β -lipotropin precursor^{16–21} (ACTH- β -LPH precursor). The primary structure of human preproenkephalin B has been deduced from the gene sequence. The amino acid sequence homology observed between preproenkephalin B and preproenkephalin A, together with the similarity between their gene organizations, suggests that the two genes have been generated from a common ancestor by gene duplication.

A human genomic DNA library²² was screened for phage carrying preproenkephalin B (alternatively designated prepro-dynorphin) gene sequences by hybridization *in situ*²³ at 60 °C with a porcine cDNA probe¹. To examine whether the initial phage clones thus obtained carried the entire mRNA-coding sequence, we undertook to clone cDNA for an upstream region of porcine and human preproenkephalin B mRNA; the porcine

cDNA clones isolated previously¹ included no more than 26 nucleotides of the 5'-untranslated region. A specific oligodeoxynucleotide primer complementary to a portion of the porcine or human preproenkephalin B mRNA sequence was elongated by reverse transcription of the mRNA, and the resulting cDNA transcripts were cloned in plasmid pBR322 and screened by hybridization with an appropriate restriction fragment derived from initially isolated DNA clones. Blot hybridization analysis²⁴ with these cDNA probes showed that the phage clones isolated initially did not contain the entire mRNA-coding sequence. Therefore, we further screened the human genomic DNA library using a hybridization probe derived from the human cDNA to clone a DNA segment extending upstream of the DNA inserts of the initial phage clones (Fig. 1).

Blot hybridization analysis²⁴ of human placental cellular DNA at 60–65 °C with human genomic DNA and porcine cDNA probes showed hybridization-positive fragments anticipated from the restriction map of the cloned genomic DNA (Fig. 1a), thus confirming that the cloned DNA segment containing the preproenkephalin B gene retained the sequence organization found in the cellular DNA. (The *EcoRI* site at the 5' end of the restriction map in Fig. 1a derives from the linker.) The DNA fragments containing mRNA-coding sequences were subjected to detailed restriction mapping and to nucleotide sequence analysis by the Maxam and Gilbert procedure²⁵ (Fig. 1b–e).

By comparing the human genomic DNA sequence determined with the porcine and human cDNA sequences (Fig. 2) we located three introns (intron A, intron B and intron C in the 5' to 3' direction) in the human preproenkephalin B gene. Intron A (~1.2 kilobase pairs (kb)) and intron B (~9.9 kb) are inserted within the segment encoding the 5'-untranslated region of the mRNA, 77 base pairs (bp) and 17 bp upstream from the translational initiation site, respectively. Intron C (~1.7 kb) interrupts the protein-coding sequence between the triplets encoding amino acid residues 43 and 44 of preproenkephalin B. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes²⁶. The DNA segment encoding amino acid residues 44–254 and the 1,566-nucleotide (excluding the redundant A residue) 3'-untranslated sequence of the mRNA is uninterrupted.

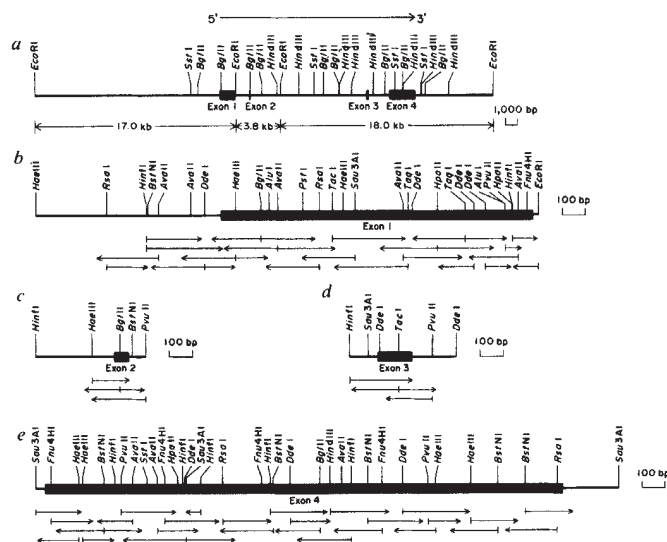
Attempts to identify the 5' terminus of preproenkephalin B mRNA on the cloned genomic DNA by the S₁ nuclease mapping procedure or by a primer elongation experiment¹⁵ were unsuccessful, apparently because the human hypothalamic poly(A) RNA preparations made available to us had a very low content of this mRNA. Because sequences homologous with a TATA box^{27,28} and a CAAT box²⁹ are found 269 bp and 305 bp upstream from the 5' end of the cloned human cDNA, respectively, and because eukaryotic mRNAs generally start with an A residue²⁸, we tentatively assign the capping site to the A residue 25 bp downstream from the putative TATA box. Although the 5'-terminal segment preceding intron A might contain an additional intron(s)²⁶, this segment is tentatively referred to as exon 1, assuming that it is uninterrupted. The following three exons are referred to as exon 2, exon 3 and exon 4 in the 5' to 3' direction.

Comparing the nucleotide sequences of the human and porcine preproenkephalin B cDNAs shows that the 60-bp human DNA sequence corresponding to exon 2 is totally absent from the porcine cDNA. Either the exon has been deleted from the porcine gene or the exonic sequence has been eliminated by RNA splicing. However, the possible existence of a human mRNA species devoid of this exonic sequence cannot be excluded.

The 5'-flanking region of the human preproenkephalin B gene contains triple tandem-repeated sequences of 68 bp (81–148 bp, 149–216 bp and 217–284 bp upstream from the putative capping site); one of the three repeats has one mismatch. The 72-bp tandem-repeated sequence in simian virus 40 and Moloney murine sarcoma virus plays an essential role as an enhancer or activator element in viral gene expression^{30–33}, although the

Fig. 1 Restriction mapping of cloned human genomic DNA containing the preproenkephalin B gene and sequencing strategy. Restriction maps with scales on the right side are given for the DNA segment composed of the 17.0-, 3.8- and 18.0-kb *EcoRI* fragments (a), part of the 17.0-kb *EcoRI* fragment containing exon 1 (b), part of the 3.8-kb *EcoRI* fragment containing exon 2 (c), part of the 18.0-kb *EcoRI* fragment containing exon 3 (d) or exon 4 (e). The direction of transcription is from left to right. For reference, the locations of the exons (see the text) are shown by closed boxes. All existing sites for the restriction endonucleases indicated are displayed in a, except for the ~13.2-kb segment located upstream of the 5'-terminal *SsrI* site and the ~5.8-kb segment located downstream of the 3'-terminal *BglII* site; only the relevant restriction sites are given in b-e. The direction and extent of sequence determinations are shown by horizontal arrows beneath the restriction maps in b-e; the sites of 5'-end labelling are indicated by short vertical lines at the end of the arrows.

Methods: The human genomic DNA library kindly provided by Dr T. Maniatis comprised a collection of recombinant phage carrying human fetal liver DNA fragments generated by partial digestion with *HaeIII* and *AluI* and joined to the λ Charon 4A arms with *EcoRI* linkers²². The hybridization probe used was the *PstI*-*PvuII* fragment of 1,165 bp containing nucleotide residues 593-1,741 of the porcine cDNA clone pLEN47¹ labelled by nick-translation³⁹ with [α -³²P]dCTP; the *PstI* site joined the cDNA to the vector⁴⁰. Three hybridization-positive phage clones were isolated from ~10⁶ plaques by repeated plaque purification. Two of these clones (first type) carried a DNA insert composed of two *EcoRI* fragments of ~3.6 and ~11.0 kb, and only the 11.0-kb fragment hybridized with the cDNA probe mentioned above; the *EcoRI* site between these two fragments represents that between the 3.8-kb and 18.0-kb fragments in a. The DNA insert of the remaining clone (second type) consisted of three *EcoRI* fragments of approximately 9.5 kb (representing the 3'-terminal portion of the 18.0-kb fragment in a), 2.6 kb and 3.4 kb, and only the 9.5-kb fragment was hybridization-positive. Two oligodeoxyribonucleotides, 5'-GCACCTCTCCCACT-3' and 5'-GCAGCCTGGCATTCG-3', which are complementary to the porcine mRNA sequence composed of residues 173-186¹ and to the human mRNA sequence composed of residues 141-155 (numbered beginning with the first nucleotide of the codon specifying the initiator methionine), respectively, were synthesized by the triester method⁴¹ and used for specific priming of the reverse transcription of porcine and human preproenkephalin B mRNA. Single-stranded cDNAs were synthesized using these primers and porcine or human hypothalamic poly(A) RNA^{42,43} as template and were converted to double-stranded cDNAs, which were cloned in plasmid pBR322 by inserting them into the *PstI* site using poly(dG)-poly(dC) homopolymeric extensions; the procedures used were as described previously³⁶, and human hypothalami were obtained at autopsy. The resulting cDNA clones were screened⁴⁴ by hybridization with the 5'-end-labelled *Fnu4HI*(20)-*HpaII*(72) fragment derived from the porcine cDNA clone pLEN179¹ or with the nick-translated 273-bp *Sau3AI*-*PvuII* fragment derived from the human genomic DNA containing exon 3 (in d). Fifteen hybridization-positive porcine cDNA clones were isolated from ~6 × 10⁴ transformants, and three hybridization-positive human cDNA clones from ~4 × 10⁵ transformants. One of the porcine cDNA clones, pLEN222, and one of the human cDNA clones, pLEN13, which apparently carried the largest cDNA insert, were subjected to DNA sequencing²³. Clone pLEN222 carried a cDNA sequence composed of residues -282 to 114, and clone pLEN13 harboured a cDNA sequence composed of residues -277 to 136; the negative numbers indicate residue numbers in the 5'-untranslated sequence beginning with the residue immediately preceding the translational initiation site. The cDNA insert of clone pLEN13 hybridized with both the 3.6-kb and the 11.0-kb *EcoRI* fragment of the phage clone of the first type. Further screening of ~10⁶ plaques from the human genomic DNA library to hybridization with the nick-translated ~280-bp *PstI*-*HpaII* fragment (containing residues -19 to -277) derived from the 5'-terminal portion of clone pLEN13 yielded two hybridization-positive clones. One of these clones (third type) carried a DNA insert consisting of two *EcoRI* fragments with approximate lengths of 17.0 kb (representing the 17.0-kb fragment in a) and 2.0 kb oriented in the 5' to 3' direction; both these fragments hybridized with the human cDNA probe. The 3.6-, 9.5-, 11.0- and 17.0-kb *EcoRI* fragments in the phage clones were subcloned⁴⁵ in plasmid pBR322 for further analysis. For cloning human cDNA for a further upstream region, ~5 pmol of the 57-bp *PvuII*-*AvaII* fragment (5'-end labelled at the *AvaII* site), derived from the 3'-terminal region of exon 1 (in b), was denatured at 80 °C for 5 min in the presence of 70% formamide, 0.4 M NaCl and 90 μ g of human hypothalamic poly(A) RNA; the total volume was 0.2 ml. Hybridization was allowed to proceed at 50 °C overnight. The DNA-RNA hybrid, collected by ethanol precipitation, was used for cDNA synthesis, and the resulting cDNA was cloned in plasmid pBR322 as described above. Screening of ~10⁵ transformants by hybridization with the nick-translated 1,033-bp *BglII*-*PvuII* fragment corresponding to most of exon 1 (in b) yielded seven hybridization-positive clones, which were subjected to DNA sequencing²³. These clones carried cDNA sequences composed of the following residues: pLEN23 and pLEN24 (residues -273 to -371); pLEN25 (residues -309 to -460); pLEN40 and pLEN41 (residues -471 to -601); pLEN 28 (residues -644 to -1,117); pLEN39 (residues -825 to -1,148).



repeats in the two viruses exhibit no detectable sequence homology with each other. There is no apparent homology kephalin B gene and the viral repeated sequences. It is tempting to hypothesize that the tandem-repeated sequence preceding this cellular gene is involved in the modulation of gene expression.

The complete primary structure of human preproenkephalin B has been deduced from the corresponding gene sequence (Fig. 2). The translational initiation site assigned is supported by the presence of a nonsense codon (TGA) found in frame 7-9 nucleotides upstream from this site in the human sequence. Human preproenkephalin B is composed of 254 amino acids including a putative signal peptide^{34,35} of 20 amino acids¹. It exhibits two amino acid deletions (Asp-Lys between residues 197 and 198) in comparison with its porcine counterpart. The deduced amino acid sequences of human neo-endorphin, dynorphin and rimorphin (also called dynorphin B⁹) are identical with the sequences of their porcine counterparts, whereas the deduced sequence of the carboxy-terminal portion of human leumorphin differs in three amino acid substitutions from porcine leumorphin: that is, Ser (residue 248) instead of Tyr, Gly (residue 249) instead of Glu, and Ala (residue 254) instead of Val.

We have previously noted that preproenkephalin B¹, preproenkephalin A (also called preproenkephalin)³⁶ and the ACTH- β -LPH precursor (also called preproopiomelanocortin)³⁷ have

almost the same numbers of amino acids and similar structural organizations with multiple repetitive units (enkephalin or melanotropin sequences) in the carboxy-terminal one- or two-thirds of their molecules and a cysteine-containing amino-terminal sequence preceded by a signal peptide^{1,36}. We have also observed considerable amino acid sequence homology between preproenkephalin B and preproenkephalin A, including the six conserved cysteine residues present at almost equivalent positions in the amino-terminal region of the prohormones¹. In Fig. 3, the amino acid sequences of porcine and human preproenkephalin B are aligned with those of human and bovine preproenkephalin A. Sequence homology extends over the entire molecules. Of the positions aligned, pairs of identical or chemically similar amino acids occupy 55% of porcine preproenkephalin B/human preproenkephalin A, 58% of porcine preproenkephalin B/bovine preproenkephalin A, 52% of human preproenkephalin B/human preproenkephalin A and 54% of human preproenkephalin B/bovine preproenkephalin A; gaps have been counted as one substitution regardless of their length. The observed extents of similarity are statistically significant (Fig. 3). This, together with the similarity found between the structural organizations of the genes encoding the two precursors (see below), suggests that the preproenkephalin A and preproenkephalin B genes originated from a common ancestor by gene duplication.

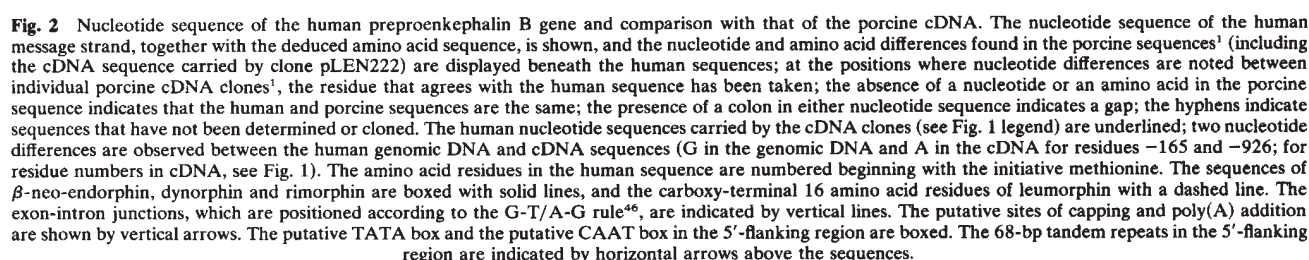


Fig. 3 Alignment of the amino acid sequences of porcine (top) and human (second row) preproenkephalin B with those of human (third row) and bovine (bottom) preproenkephalin A. The one-letter amino acid notation is used. Sets of four identical residues are enclosed with solid lines, and sets of four residues that are identical or are considered to be favoured amino acid substitutions are enclosed with dotted lines. Favoured amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W⁴⁷. Gaps (-) have been inserted to achieve maximum similarity. Positions in the aligned sequences including gaps are numbered beginning with that of the initiative methionine. The locations of the enkephalin sequences are indicated by thick lines above the preproenkephalin B sequences and below the preproenkephalin A sequences. The position at which the protein-coding sequence is interrupted by an intron is shown by an arrow. The extent of similarity between the sequences was assessed statistically, as described previously⁴⁸. In these tests, the positions corresponding to the enkephalin and extended enkephalin sequences and the paired basic amino acid residues flanking them (positions 105–121, 142–150, 197–209, 231–239, 251–259 and 283–291) were excluded from both of the sequences compared. The alignment score A^{48} and the probability (P) that the score is realized by chance were evaluated for each pair of the aligned preproenkephalin A and preproenkephalin B sequences as follows: porcine preproenkephalin B/human preproenkephalin A, $A = 5.48$ and $P < 10^{-4}$; porcine preproenkephalin B/bovine preproenkephalin A, $A = 5.45$ and $P < 10^{-4}$; human preproenkephalin B/human preproenkephalin A, $A = 2.90$ and $P = 1.9 \times 10^{-3}$; human preproenkephalin B/bovine preproenkephalin A, $A = 5.26$ and $P < 10^{-4}$. The sequence data for porcine preproenkephalin B have been taken from ref. 1, and those for bovine and human preproenkephalin A from ref. 36 and refs 15 and 49, respectively.

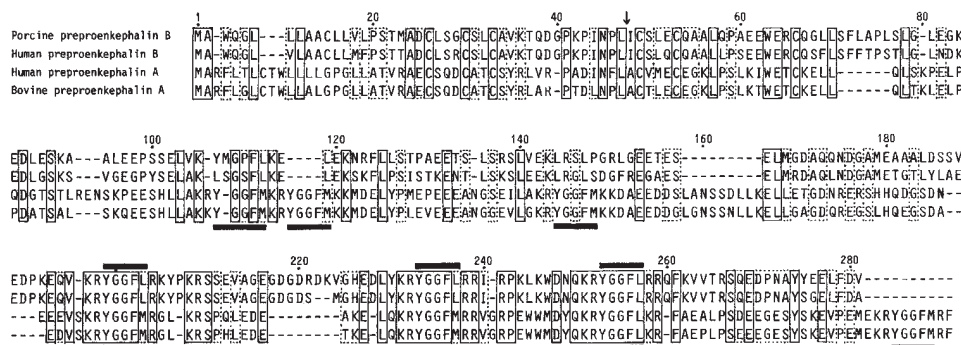


Figure 4 shows the striking similarity among the structural organizations of the mammalian genes encoding preproenkephalin B, preproenkephalin A and the ACTH- β -LPH precursor, which all represent a multi-hormone precursor^{1,36–38}. All three genes contain an intron in the protein-coding sequence near the signal peptide region and another intron in the segment

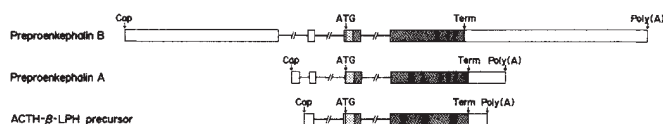


Fig. 4 Schematic representation of the structural organizations of the mammalian genes encoding preproenkephalin B (top), preproenkephalin A (middle) and the ACTH- β -LPH precursor (bottom). Exons are shown by blocks, and introns by lines; the lengths of the lines do not always represent the real lengths of the introns. The sites of capping, translational initiation (ATG), translational termination (Term) and poly(A) addition are indicated; the capping site of the preproenkephalin B gene (human) has been tentatively assigned, and the possibility that the 5'-terminal region of this gene contains an additional intron(s) cannot be excluded (see the text). The coding regions for the repeated enkephalin or melanotropin structures are indicated by closed boxes, those for the signal peptide by stippled boxes, and the remaining protein-coding regions by hatched boxes. The data for the preproenkephalin A gene (human) have been taken from ref. 15, and those for the ACTH- β -LPH precursor gene (bovine, human and mouse) from refs 16–21; a minor mRNA species that contains the intronic sequence between the 5'-terminal two exons of the human preproenkephalin A gene has been detected¹⁵.

encoding the 5'-untranslated sequence near the translational initiation site; the protein-coding sequences of the preproenkephalin A and preproenkephalin B genes are split at an equivalent position (see Fig. 3). Thus, all of the repeated enkephalin or melanotropin sequences contained in these precursors are encoded by a single large exon. A second small exon of similar size encodes the signal peptide. The observed resemblance in structural organization suggests that the preproenkephalin A and preproenkephalin B genes and the ACTH- β -LPH precursor gene may have evolved by an analogous mechanism^{15,38}.

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Correlation between a DNA restriction fragment length polymorphism and C4A6 protein

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The fourth component of complement (C4) in man, is coded for by two separate but closely linked loci (*C4A* and *C4B*) within the major histocompatibility region (MHC), on the short arm of chromosome 6¹. Like class I and II loci of this region, the *C4* genes are highly polymorphic with more than 30 alleles, including null alleles, assigned to the two loci². This extensive polymorphism, based mainly on electrophoretic mobility, provides a useful marker for studies of disease susceptibility. Several disorders, including systemic lupus erythematosus and type I diabetes^{3,4}, show associations with C4 phenotypes. We have used the technique of Southern⁵ with a *C4* specific probe⁶ to examine the genomic DNA of individuals typed for C4 by protein electrophoresis. We have identified 10.7 and 3.8 kilobase (kb) *Bgl*III restriction fragments in each of 9 unrelated individuals with a *C4A6*^{7,8} allele, and in none of 22 unrelated individuals in whom this allele was not expressed. This clear correlation of restriction fragment length polymorphism with C4 phenotype provides a precise basis for analysis of C4 polymorphism. It is likely to be of value in clinical investigations of autoimmune disease.

Genomic DNA was prepared from peripheral blood of individuals typed for C4 and samples were digested with various restriction endonucleases, the resulting fragments separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with a ³²P-labelled cDNA probe known to hybridize with both *C4* loci (Fig. 2b). The two cDNA probes used, *Alu-7* and clone F, are described in Fig. 3.

Figure 1 shows the results of Southern analysis of 12 DNA samples using the restriction endonuclease *Bgl*III and the clone F cDNA probe. A 14.5 kb fragment was common to all individuals and therefore is probably part of both the *C4A* and *C4B* loci. The extra 10.7 and 3.8 (kb) fragments, were present only in individuals typed positive for the *C4A6* protein. Of 31 unrelated individuals examined, all 9 expressing *C4A6* showed the extra 10.7 and 3.8 kb bands whereas the 22 not expressing the *C4A6* protein did not show the two extra bands. The two extra bands suggest the *C4A6* allele has an extra *Bgl*III site.

We confirmed the segregation of the 10.7 and 3.8 kb *Bgl*III

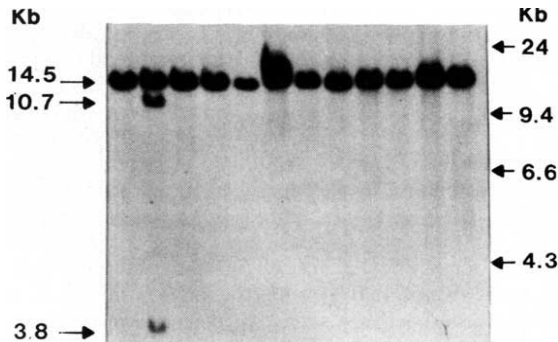


Fig. 1 The *C4A6* allele has an additional *Bgl*III site. DNA from 12 unrelated individuals typed for C4 protein (described in Fig. 2 legend) was digested with the restriction endonuclease *Bgl*III according to the manufacturers suggested conditions (Boehringer-Mannheim) and analysed by Southern technique (described in Fig. 2 legend) using the *C4* cDNA probe, clone F. All samples show the common 14.5 kb band. Sample number 2 typed positive for A6 protein, second from left, shows two extra bands at 10.7 and 3.8 kb.

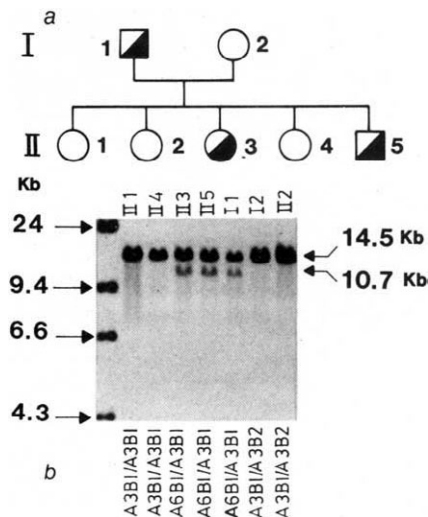


Fig. 2 a, Pedigree of family J. F. □ male; ○ female; half-shaded, heterozygous for *C4A6* allele. b, Southern analysis of family J. F. using the restriction endonuclease *Bgl*III and hybridizing with *C4* cDNA probe, *Alu-7*. All individuals show the common 14.5 kb band. The father (I 1) and two children (II 3 and II 5) typed as *C4A6* each have the extra 10.7 kb band. Other family members not typed as *C4A6* did not have the extra 10.7 kb fragment.

Methods: a, Plasma from individuals was typed for C4 using the procedure described by Awdeh and Alper⁸. Briefly EDTA plasma was treated overnight with neuraminidase (Sigma type VI) then separated on 0.8% agarose gels using Tris-glycine-barbital buffer, pH 8.8. C4 bands were visualized by overlaying with goat anti-human C4 antisera (Atlantic Antibodies), elution of uncomplexed protein by washing in saline and finally staining immunoprecipitates with Coomassie blue. b, Genomic DNA was prepared from whole blood by the procedure of Sykes (see ref. 10). Briefly, after lysis of cells in 0.9% NaCl/0.1% Nonidet P40, pellets were broken up in lysis buffer (7 M urea, 0.3 M NaCl, 10 mM EDTA, 10 mM Tris HCl-pH 7.5) with a glass rod, and incubated at 37 °C for 10 min after the addition of 2% SDS. DNA was extracted three times with phenol/isoamylalcohol/chloroform (45:10:45) followed by precipitation by ethanol and dialysis against 10 mM Tris-HCl pH 7.5, 1 mM EDTA. For Southern analysis, 8–10 µg DNA samples were digested for 20 h at 37 °C in a final volume of 50 µl with 18 units of *Bgl*III restriction endonuclease according to the manufacturer's specifications (Boehringer-Mannheim), electrophoresed in 0.8% agarose and transferred to nitrocellulose filters (Sartorius). Nitrocellulose filters were prehybridized at 42 °C for at least 2 h in 50% formamide, 1 M NaCl, 0.01 M EDTA, 10x Denhardt's solution, 10% dextran sulphate, 50 mM Tris-HCl pH 7.5, 0.1% SDS, 0.1% sodium pyrophosphate and 0.1 mg ml⁻¹ heat-denatured herring sperm DNA and then hybridized with nick-translated (*Amersham International*), heat-denatured cDNA probes with a specific activity of 10⁸ c.p.m. µg⁻¹ (0.5 µCi ml⁻¹) for 24–48 h⁹. After hybridization, filters were washed six times for 10 min in 2×SSC, 0.1% SDS (1×SSC = 0.15 M NaCl, 0.015 Na citrate) and six times for 10 min at 68 °C in 1×SSC, 0.1% SDS. After drying, filters were exposed to X-ray film with an intensifying screen at -70 °C for 2 days.

fragments with the A6 allele by studying a family in which the father and two children expressed the A6 allele. Figure 2a shows that of four children two (II 3 and II 5) inherited both the A6 allele and the 10.7 kb fragment from their father (I 1) (Fig. 2b). The 3.8 kb fragment was not seen because the cDNA probe used, *Alu-7*, did not hybridize with this region. Of 10 other restriction endonucleases tested (*Eco*RI, *Kpn*I, *Acc*I, *Sst*I, *Xba*I, *Hind*III, *Xho*I, *Pst*I, *Hpa*I and *Hinf*I), none showed differences related to the A6 allotype.

The origin of the two extra *Bgl*III fragments was determined by mapping the common *Bgl*III sites within a cloned genomic fragment. This cloned fragment probably contains a complete *C4* gene and has been mapped using both the *Alu-7* and clone F cDNA probes (see Fig. 3 legend). An additional *Bgl*III site 3.8 kb downstream from the 5' end of the 14.5 kb *Bgl*III fragment (Fig. 3) would convert the 14.5 kb fragment to the 3.8

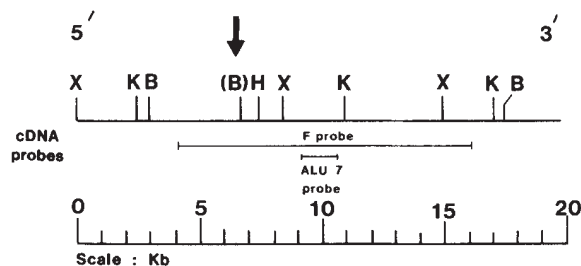


Fig. 3 Line diagram of a limited restriction map of 20 kb of a cloned genomic fragment containing a *C4* gene. This represents further characterization of a cosmid fragment reported earlier⁶. The arrow indicates a possible site for an additional *Bgl*II site in the *A6* allele which would convert the common 14.5 kb *Bgl*II fragment into two fragments of 10.7 and 3.8 kb. Both probes used are cloned *C4* cDNA inserts. *Alu-7* is a 304 bp fragment previously described, which hybridizes to the *C4d* region of the *C4* mRNA⁶, while the 4.8 kb clone *F* probe extends from the 3' non-coding region to within approximately 400 bp from the 5' end of the coding region (T. Belt and M.C.C., unpublished) (B, *Bgl*II; H, *Hind*III; K, *Kpn*I; X, *Xho*I).

and 10.7 kb fragments seen associated with the *A6* allele. However, all three *Bgl*II fragments (14.5, 10.7 and 3.8 kb) would still be seen since the 14.5 kb band is common to both *C4A* and *C4B* loci. It is not known if the additional *Bgl*II site of the *A6* allele is in the coding region of the *C4* locus.

During this study many other restriction fragment length polymorphisms were found in the *C4* loci but only the *Bgl*II digest gave a simple correlation with a protein polymorphism. Extensive sequencing of the *C4* genes will be necessary to establish the full extent of the polymorphism which occurs in this complement protein. Present evidence suggests that it is likely to be comparable to that found in the MHC class I and II proteins.

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Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics

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Conjugative plasmids were common in enterobacteria isolated before the medical use of antibiotics. Plasmid F of *Escherichia coli* K-12 was one example and we¹ identified others in over 20% of a collection of strains isolated between 1917 and 1954, the Murray collection. In the past 25 years, conjugative plasmids encoding antibiotic resistances have become common in

bacteria of the same genera as those of the Murray Collection—*Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Escherichia*. The present study was made to show whether the 'pre-antibiotic' plasmids belonged to the same groups, as defined by incompatibility tests (Inc groups), as modern R plasmids. Of 84 such plasmids established in *E. coli* K-12, none with antibiotic resistance determinants, 65 belonged to the same groups as present resistance (R) plasmids. Thus the remarkable way in which medically important bacteria have acquired antibiotic resistance in the past 25 years seems to have been by the insertion of new genes into existing plasmids rather than by the spread of previously rare plasmids.

Among a collection of Enterobacteriaceae made by E. D. G. Murray between 1917 and 1954, 84 (19%) transferred conjugative plasmids to *E. coli* K-12. Of these, 36 determined colicinogeny, one determined tellurite resistance and 47 were cryptic¹. We labelled cryptic conjugative plasmids with antibiotic resistance transposons, converting them to R plasmids, as follows. The cryptic plasmids had been identified by their ability to mobilize a non-conjugative R plasmid, R300B (streptomycin and sulphonamide resistance) or pHH1310a (ampicillin resistance) to *E. coli* K-12 F[–] and thence between nutritionally distinguishable lines of K-12. We now used them to mobilize R300B or pHH1310a to K-12 strains with chromosomally located transposons and selected in further matings for transfer of the transposon-encoded resistance. Some transconjugants which acquired the transposon also acquired R300B or pHH1310a. Irrespective of this, all were tested for their ability to transfer the transposon-encoded resistance to a further K-12 recipient. If they did so, labelling of the cryptic conjugative plasmid was implied and was confirmed by showing that the plasmid DNA had increased in molecular weight by the amount expected. Plasmid molecular weights were estimated after agarose gel electrophoresis of lysates as previously described¹.

We used transposons Tn5 (kanamycin resistance), Tn7 (trimethoprim and streptomycin/spectinomycin resistance) and Tn9 (chloramphenicol resistance)² from AB1157::Tn5, J62::Tn7 and JC3272::Tn9. Attempts were made to label each of the 47 plasmids with each of the transposons Tn7 and Tn9. Twenty-one plasmids were labelled with each, 10 with Tn7 but not Tn9 and 5 with Tn9, but not Tn7. When first labelling attempts failed they were repeated and Tn5 was also used. Five plasmids accepted Tn5 but not Tn7 or Tn9. In all, 46 of the 47 plasmids mobilized one or more transposons, bringing about their transfer into another strain. Forty-one plasmids were labelled, that is, the transposon was inserted in the plasmid, the other five were lost at this stage.

The conjugative plasmids were tested for stable co-existence in *E. coli* K-12 (compatibility) with a range of standard plasmids of 21 known groups and 4 plasmids belonging to no known group. Compatibility was demonstrated not only as stable co-inheritance of the respective plasmid markers but also by identification of both plasmid species in lysates and by showing their ability to be separately transferred to another K-12 strain. Incompatibility was indicated by elimination of the resident plasmid when the second plasmid was introduced by conjugation or, if coexistence did occur, by the rapid segregation of the two plasmids into separate clones during bacterial growth.

Sixty-five 'pre-antibiotic' plasmids belonged to one of seven of the R plasmid Inc groups defined since 1971 (Table 1). Among the remaining 13 with recognizable labels, 7 fell into two 'new' Inc groups, that is groups that have not been identified among R plasmids. There were five plasmids in one of these groups and two in the other. Six plasmids were compatible with all standard plasmids, with members of the new groups and with one another. None was incompatible with any of the four previously unclassified plasmids.

We do not have full details of sources of the Murray strain collection but the large number of IncI₁ plasmids seems likely to be biased by the inclusion of multiple examples of isolates from outbreaks of *Shigella sonnei* infection in 1937 and *Salmonella typhimurium* in 1941. Counting only one example from

Table 1 Classification of 'preantibiotic' conjugative plasmids

Inc group	No. of plasmids	Molecular weight ($\times 10^6$)*	Marker†	Origin‡
B	2	50	Tn5, Tn7	<i>Kleb</i> '40 <i>ShigS</i> '33
FII	3 {1	50	ColB	<i>S.sp</i> '40
	2 {2	35	Tn7, Tn9	<i>S.sp</i> '39
FIV	4	35	Tn5, Tn7, Tn9	<i>E.c</i> '34 2? <i>Shig</i> ?
		45–50	Tn5, Tn7	<i>S.sp</i> '27'28 '37
I ₁	38 {3		ColI	<i>ShigS</i> 2'37 <i>ShigF</i> ?
	20 {3	55–60	ColIa	<i>ShigS</i> 15'37'39'54, ? <i>S.tm</i> '35 <i>S.sp</i> '38
	12 {12		ColIb	<i>S.tm</i> '35 10'41 <i>S.sp</i> '38
I ₂	1	40	Tn7	<i>E.c</i> ?
N	2	25	Tn7, Tn9	<i>S.sp</i> '30 <i>Kleb</i> ?
X	15	30	Tn5, Tn7, Tn9	{ <i>S.ty</i> '35'36'37'38'39 <i>S.para</i> '25 <i>S.ent</i> '34 <i>S.tm</i> '17'31 <i>S.sp</i> 2'26'28'29 <i>Kleb</i> '32'40
Murray I	5	25–35	Tn5, Tn7, Tn9	<i>ShigS</i> '20'27 <i>ShigF</i> '18 <i>E.c</i> '34 <i>S.sp</i> '35
Murray II	2	n.m.	Tn7, Tn9	<i>ShigD</i> '32 <i>S.sp</i> '36
Unclassified	6 {1	105	Tellurite R	<i>Kleb</i> '40
	5 {5	30–40	Tn7, Tn9	<i>ShigS</i> '37, ? <i>S.sp</i> '35 2'40

Conjugative plasmids were first tested for compatibility with standard plasmids of Inc groups B, C, FI, FII, HI, I₁, I₂, J, K, M, N, P, T, W and X¹². No dual incompatibility was seen. Plasmids compatible with these were tested with examples of Inc groups FIV¹², HII¹³, U¹⁴, D¹⁵, V¹² and Y¹² and with individual plasmids Folac¹⁵, R441¹⁶, R775¹⁵ and R71a¹⁵. Those still unclassified were tested in pairs, each against all. For the purpose of this report we have called the Inc groups so defined Murray I and Murray II.

* Molecular weights before transposon-labelling and rounded to nearest 5×10^6 . n.m., Not measured.

† Colicins were identified using sensitive indicator strain *E. coli* K-12 Row, colicin I resistant strain BC3 and K12 strains immune to B, Ia or Ib carrying plasmid ColB-K98, R483ColIa or R144ColIb¹².

‡ After each entry the year of collection is given. ? indicates year of collection unknown. When there is more than one entry in a year, the number is shown in italics. Abbreviations for donor bacteria: *Salmonella typhi*, *S.ty*; *Salmonella paratyphi* A, *S.para* A; *S. typhimurium*, *S.tm*; *Salmonella enteritidis*, *S.ent*; other *Salmonella* serotypes, *S.sp*; *Shigella dysenteriae*, *ShigD*; *Shigella flexneri*, *ShigF*; *S. sonnei*, *ShigS*; unspiciated *Shigella*, *Shig*; *Klebsiella*, *Kleb*; *E. coli*, *E.c*.

each of these supposed outbreaks, we are left with 53 conjugative plasmids with markers appropriate for Inc testing, of which 40 (75%) belonged to known Inc groups. In classifying R plasmids from the same genera currently isolated, a higher proportion classifiable into standard groups would be expected^{3–6}. No plasmids of Inc groups C, H, M, P or W, now widely distributed, were found in this collection. High titre preparations of phages PR4, MS2 and I_{ke}, that use conjugative pili as receptors^{7–9} were spotted on K-12 strains carrying each plasmid; three plasmids of the Murray I group determined sensitivity to phage PR4, sensitivity that is also conferred by plasmids of IncP and IncW. The two cryptic plasmids of IncFII determined susceptibility to lysis by phage MS2, implying constitutive production of F pili, and the two IncN plasmids gave I_{ke} susceptibility.

Evidently the range and distribution of conjugative plasmids in the 1917–54 strains are not identical to those of R plasmids recently classified. Variation over this length of time might be expected, even in the absence of the powerful selective effect of antibiotic use that now encourages the dissemination of R plasmids. The selection must change distributions by favouring particular plasmids, such as those of IncFII_{me}, now common in enteric pathogens in widely separated parts of the world¹⁰.

The frequency of plasmids in these old strains and the high proportion of them that belong to the same groups as today's R plasmids indicate that the lineage of most of the latter is from plasmids resident in enterobacteria before antibiotics were used in medicine. The study of R plasmids has played a major part in revealing the evolutionary importance of genes carried in moveable, or transposable, DNA sequences. The acquisition of resistance genes by plasmids has been by the accumulation of transposons, a mode of evolution recognized as having analogies in many eukaryotes as well as bacteria¹¹.

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Diminished *in vitro* tyrosine kinase activity of the EGF receptor of senescent human fibroblasts

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Fibroblastic cultures derived from normal human tissues undergo a finite number of population doublings when serially subcultured *in vitro* (see refs 1, 2 for reviews). Epidermal growth factor (EGF) serves as a mitogen for early doubling level cultures of the human fetal lung-derived cell strain, WI-38, under serum-free conditions³. The ability of cells from late doubling level cultures to respond mitogenically to EGF is lost, however, despite undiminished binding of EGF throughout the replicative lifespan³. The ultimate effects of EGF, that is DNA synthesis and mitosis (see ref. 4 for review), occur after a sequence of events initiated by binding of ligand to specific cellular receptors^{5,6}. The receptor for EGF has been characterized as a 145,000–165,000 (145 K–165 K) molecular weight doublet^{7,8}, and, like the receptors for platelet-derived growth factor⁹ and insulin^{10,11}, and the transforming proteins of certain of the RNA tumour viruses^{12–17}, is a tyrosine-specific protein kinase with autophosphorylating activity^{8,18,19}. Moreover, several of the cellular target molecules of tyrosine phosphorylation have been found to be substrates for two or more of these

kinases^{20,21}. The hypothesis that tyrosine phosphorylation underlies a common mechanism of growth control prompted us to ask whether the loss of responsiveness to EGF by late doubling level WI-38 cells is accompanied by altered expression of the EGF receptor, and specifically whether changes occur in the ability of receptors from populations of cells of various *in vitro* ages to catalyse tyrosine autophosphorylation. We show here that autophosphorylating activity is absent from the EGF receptor of cells which have lost their mitogenic responsiveness to EGF.

We first related the ability of EGF to stimulate DNA synthesis with expression of EGF receptors by WI-38 cells of various *in vitro* ages. The *in vitro* age of WI-38 cultures was monitored using two indices, population doubling level (PDL)²² and per cent of the replicative lifespan completed (PLC)²³; WI-38 cells were considered young if 50% or less of the replicative lifespan was complete, and senescent if 85% or more of the replicative lifespan was complete. Density-arrested cultures were refed with a serum-free medium in which EGF was the primary mitogen^{3,24,25}, and DNA synthesis was monitored by measuring incorporation of ³H-TdR by nuclei during a 24-h pulse. In these conditions, 31% of the cells from a culture at PDL 40, which had completed 49% of its replicative lifespan, incorporated label, whereas only 5% of the nuclei of cells from a culture at PDL 71, with 91% of the replicative lifespan completed, were labelled. The expression of EGF receptors by cells from replicate

cultures was assessed by specific binding of ¹²⁵I-EGF, and by determining the reactivity of an antiserum (cl 21 antiserum) specific for the human EGF receptor⁸ with viable cells in a radioimmunoassay (RIA). While results from both assays (Table 1) reveal that cells from a culture at PDL 71 possess more specific binding sites for EGF than an equivalent number of cells from a culture at PDL 40, the number of EGF receptors is relatively constant throughout the replicative lifespan when considered as a function of cell surface area (see also ref. 3).

To compare the EGF receptors from young and senescent cultures, we adjusted volumes of cell extracts so that samples contained equal numbers of cl 21 antibody binding sites as determined by RIA of replicate cultures. Inherent autophosphorylating activity was assessed by incubating cl 21 antiserum-EGF receptor immune complexes with [γ -³²P]ATP and identifying proteins that incorporated isotope after SDS-PAGE and autoradiography. We found a striking difference in the level of this activity depending on the *in vitro* age of the culture: both molecular weight forms of the receptor (145 K and 165 K) from young cells became phosphorylated in this assay (Fig. 1A), whereas autophosphorylation of receptor proteins from senescent cultures was virtually undetectable (Fig. 1B). Inclusion of EGF in the reaction mixture enhanced radiolabelling of receptors from early doubling level cultures (Fig. 1C), but did not stimulate any inherent kinase activity in receptors from senescent cells (Fig. 1D). That tyrosine is the sole substrate for the receptor kinase of young WI-38 cells was confirmed by phosphoamino acid analysis of the two molecular weight forms of the receptor purified after *in vitro* labelling of immune complexes with [γ -³²P]ATP (Fig. 2A, B).

We also compared the electrophoretic mobilities of receptor proteins from cultures of different PDLs after either metabolic labelling with ³²P_i, or cell surface labelling with ¹²⁵I by the

Fig. 1 Electrophoretic analysis of proteins immunoprecipitated by cl 21 antiserum from WI-38 cells. Panel 1: Immune complexes (see below) were resuspended in 60 μ l of a buffer containing 20 mM HEPES at pH 7.4, 0.1% bovine serum albumin, 10% (v/v) glycerol, 30 mM NaCl, and 50 mM MgCl₂¹⁸, and the kinase reaction initiated by the addition of 50 μ Ci [γ -³²P]ATP (2,000 Ci mmol⁻¹; Amersham). In certain experiments, 100 ng EGF (Collaborative Research) was added to the reaction mixture. The reaction was carried out for 5 min at 4 °C, and the immune complexes washed and proteins removed from bacteria as previously described⁴¹ for analysis by SDS-PAGE on gels prepared with 5% acrylamide. [γ -³²P]ATP labelling of immunoprecipitates from A, young (PDL 40, PLC 49%) and B, senescent (PDL 70, PLC 90%) WI-38 cells. EGF-stimulated [γ -³²P]ATP labelling of immunoprecipitates from C, young (PDL 40, PLC 50%) and D, senescent (PDL 70, PLC 90%) WI-38 cells. Panel 2: cells were rinsed 3 times with PBS and incubated with 1 mCi ³²P_i (carrier-free, 8 mCi ml⁻¹; Amersham) in 1 ml phosphate-free medium for 4 h at 37 °C in an atmosphere of 5% CO₂-95% air before extraction with NP-40. Immunoprecipitation of E, young (PDL 42, PLC 52%) and F, senescent (PDL 74, PLC 100%) cell extracts after metabolic labelling with ³²P_i. Arrows denote the 145 K and 165 K molecular weight forms of the EGF receptor. Molecular weight markers ($\times 10^{-3}$) were: myosin, 205; phosphorylase A, 92; and bovine serum albumin, 66.

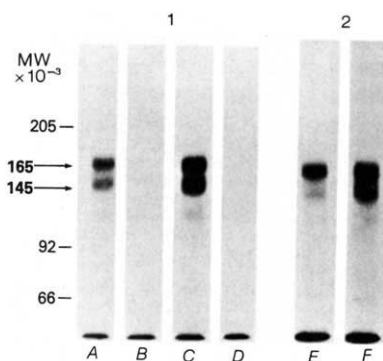
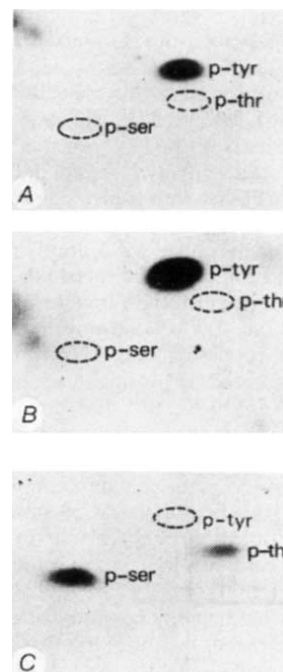


Fig. 2 Two-dimensional analysis of the phosphoamino acids of the EGF receptor from young WI-38 cells. Phosphoamino acids of the 145 K (A) and 165 K (B) molecular weight forms of the EGF receptor purified from young (PDL 40, PLC 50%) WI-38 cells after labelling of immune complexes with [γ -³²P]ATP. C, Phosphoamino acids of the 165 K molecular weight form of the EGF receptor purified from young (PDL 42, PLC 52%) after metabolic labelling with ³²P_i; identical results were obtained for the 145 K receptor protein of young cells and for both molecular weight forms of the receptor of senescent cells. Origins of samples are denoted by arrows.

Methods: Immunoprecipitated EGF receptor proteins were separated on SDS-polyacrylamide gels containing 5% acrylamide and localized by autoradiography. Bands were excised and allowed to swell in a buffer containing 0.05 M NH₄HCO₃ and 0.1% SDS. Procedures for protein recovery⁴³ and acid hydrolysis¹² are presented elsewhere. Acid hydrolysates were diluted with 250- μ l deionized H₂O and incubated at room temperature with 100 μ l of 50% suspension of DOWEX-50W cation exchange resin beads for 15 min (A. Ross, Wistar Institute; personal communication). Beads were pelleted, and the hydrolysate dried and resuspended in 5 to 10 μ l of a marker mixture containing phosphotyrosine, phosphothreonine, and phosphoserine, each at 1 mg ml⁻¹. Phosphoamino acids were resolved on cellulose thin-layer plates in two dimensions, the first by electrophoresis at pH 1.9 for 60 min at 1.5 kV; the second by ascending chromatography in isobutyric acid-0.5 M NH₄OH (5:3, v/v). ³²P-labelled amino acids were localized by autoradiography and phosphoamino acid markers by staining with ninhydrin.



Methods: Quiescent WI-38 cultures of various *in vitro* ages were rinsed, scraped, and extracted with 0.1% Nonidet P-40 (NP-40) as previously described⁴¹. The volumes of these extracts were adjusted so that samples to be compared contained equivalent numbers of cl 21 binding sites as determined by RIA (see Table 1). Cell extracts were cleared of material that binds nonspecifically to protein-A-bearing *Staphylococcus aureus* by three half-hour incubations with excess bacteria at 4 °C. Absorbed cl 21 antiserum or preimmune normal mouse serum was incubated with *S. aureus* (75 μ l of a 1:10 dilution of serum per 50 μ l of a 10% solution of bacteria) for 1 h at 4 °C. Cleared cell extracts were then incubated with antibody-bearing *S. aureus* for 1 h at 4 °C, and the resulting immune complexes washed by the method of Cullen and Schwartz⁴².

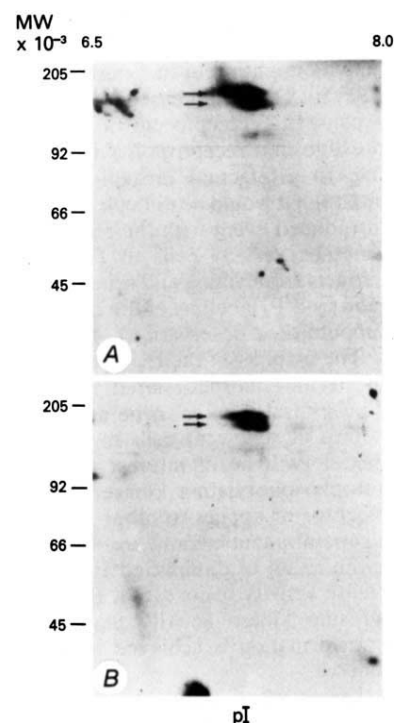
lactoperoxidase method²⁶. Both molecular weight forms of the receptor were expressed by young and senescent WI-38 cells, although there were differences in their relative abundance; when cells were metabolically labelled with ³²P_i, the 165 K receptor protein was preferentially detected relative to the 145 K receptor protein in young cells, while in senescent cells, labelling of the two molecular weight forms was essentially equivalent (Fig. 1E, F). Metabolic labelling of the EGF receptor with ³²P_i occurs chiefly at serine and threonine residues, phosphotyrosine being so minor a component as to be undetectable (Fig. 2C). The isoelectric points of ¹²⁵I-labelled receptor proteins are the same whether immunoprecipitated from cells of early or late PDL (Fig. 3), and are identical to those previously reported for human epidermoid carcinoma A431 cells⁸.

We have presented evidence here that autophosphorylating activity is essentially absent in receptors from cells no longer mitogenically responsive to EGF. This, taken with studies in which transformation-defective mutants of certain RNA tumour viruses whose transforming proteins lack *in vitro* kinase activity have been isolated^{27,28}, suggests that tyrosine phosphorylation is, at least in some cases, essential to cell proliferation. Several findings, however, argue that autophosphorylation by tyrosine kinases alone is not sufficient to elicit a proliferative response. The major tyrosine phosphorylation site (tyr-416) of pp60^{src} can be eliminated without altering the ability of the protein to phosphorylate a second internal tyrosine site as well as exogenous substrates, or to mediate cell transformation²⁹; divalent monoclonal antibodies specific for the EGF receptor inhibit binding of EGF and enhance autophosphorylation yet promote neither receptor internalization nor DNA synthesis³⁰; and, phosphorylation of exogenous synthetic substrates by the EGF receptor kinase is not dependent on receptor autophosphorylation, although the two kinase activities most probably utilize a common catalytic site³¹. We can therefore speculate that autophosphorylation by growth regulatory proteins confers competence rather than commitment to proliferate.

Several mechanisms to account for the age-dependent diminished autophosphorylation of the EGF receptor of WI-38 cells can be envisioned. The activity of the catalytic site or the availability of internal tyrosine phosphorylation sites may be altered or absent in senescent cells. Alternatively, there may be a conformational change associated with the receptor protein

Fig. 3 Two-dimensional electrophoretic analysis of proteins immunoprecipitated by cl 21 antiserum from NP-40 extracts of quiescent WI-38 cells after cell surface labelling with ¹²⁵I by the method of Hynes²⁴. **A**, Young (PDL 34, PLC 45%) and **B**, senescent (PDL 79, PLC 99%) WI-38 cells. Arrows denote the 145 K and 165 K molecular weight forms of the EGF receptor. Molecular weight markers are the same as in legend to Fig. 1 with the addition of actin, 45.

Methods: Immune complexes were removed from *S. aureus* by incubation in buffer containing 9.5 M urea, 2% (w/v) NP-40, 5% (v/v) 2-mercaptoethanol, 1.6% (w/v) pH 6-8 ampholytes and 0.4% (w/v) pH 3-10 ampholytes⁴⁴. Samples were separated in the first dimension on isoelectric focusing gels prepared with pH 6-8 ampholytes, and in the second dimension, on SDS-polyacrylamide gels containing 7.5% acrylamide by the method of O'Farrell⁴⁴.



in senescent cells. Whereas it is unlikely that native receptor conformation is preserved in immune complexes, a tightly associated membrane component resistant to detergent extraction might exist that either confers an active conformation *in vitro* or is an essential enzyme cofactor; absence or altered turnover of such an effector molecule might account for diminished receptor autophosphorylation in senescent cells. Alternatively, a tightly bound effector molecule that renders the inherent kinase inactive might be preferentially expressed in senescent cells. Finally, the inherent receptor kinase activity might be inactivated by an endogenous protease; in this case, production of a

Table 1 Detection of the EGF receptor on intact WI-38 cells as a function of *in vitro* replicative age

Cell type	% Nuclei incorporating [³ H]TdR after EGF stimulation	Moles ¹²⁵ I-EGF bound per 10 ⁵ cells	Binding of human EGF receptor antiserum in indirect RIA	Binding of preimmune normal mouse serum in indirect RIA	Mean cell surface area
WI-38 (PDL 40, PLC 49%)*	31%	9 × 10 ⁻¹⁵	2,099 (±162)	429 (±17)	650 μm ²
WI-38 (PDL 71, PLC 91%)	5%	17 × 10 ⁻¹⁵	3,116 (±333)	572 (±30)	1,154 μm ²
A431	—	—	22,068 (±775)	849 (±50)	—
MOLT-4	—	—	479 (±111)	246 (±21)	—

Cultures of WI-38 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Flow Laboratories) as previously described^{22,23}. The ability of cultures to initiate DNA synthesis in response to EGF stimulation was assessed by scoring the per cent nuclei labelled with [³H]TdR as follows. Cells were seeded onto glass coverslips, and at confluency (7 to 15 days later) refed with MCDB-104 medium³⁴ supplemented with EGF (25 ng ml⁻¹), insulin (5 μg ml⁻¹), transferrin (5 μg ml⁻¹), dexamethasone (55 ng ml⁻¹)^{3,24,25}, and [³H]TdR (1 μCi ml⁻¹; NEN). Coverslips were rinsed, fixed, and prepared for autoradiography 24 h later as previously described³. At least 400 nuclei were scored per coverslip, and all determinations were done in duplicate. Cell surface areas were calculated on the basis of cell volumes using a Coulter counter³. Specific binding of ¹²⁵I-EGF (100–200 μCi μg⁻¹; Collaborative Research) was determined as described by Phillips *et al.*³. Briefly, cells were seeded in MEM supplemented with 10% FBS and grown to a state of density arrest. Cultures were refed with serum-free MCDB-104 medium³⁴, rinsed with MCDB-104 medium containing human serum albumin (0.5 mg ml⁻¹) 24 h later, and incubated with various amounts of ¹²⁵I-EGF for 1 h at 4 °C. Cells were then rinsed, solubilized with 1 M NaOH, and aliquots of solubilized cells counted in a gamma counter. Specific binding was calculated by subtracting values obtained when ¹²⁵I-EGF binding was performed in the presence of 1,000-molar excess of unlabelled EGF. The moles of ¹²⁵I-EGF bound per 10⁵ cells was determined by Scatchard analysis³⁵. Cl 21 antiserum, which reacts specifically with the human receptor for EGF⁸, was prepared by injecting the human-mouse hybrid cl 21³⁶ into mice syngeneic with the mouse parental cells³⁷. Preimmune normal sera and cl 21 antisera were diluted 1:10 with phosphate-buffered saline (PBS) and absorbed 2 times with an equal volume of packed MC57G mouse cells³⁸ before use. Binding of cl 21 antiserum was measured by an indirect radioimmunoassay (RIA)^{37,39} in which trypsinized cells were subjected to two 1-h incubations at 4 °C, first with a 1:100 dilution of antiserum, then with protein A (Pharmacia) labelled with ¹²⁵I by the chloramine T method. Results are presented as the average c.p.m. bound per 10⁵ cells based on triplicate determinations (±s.d.). In addition to WI-38, two other cell lines were included in the RIA, A431, a human epidermoid carcinoma-derived cell line which expresses ten to twenty times the number of EGF receptors as normal fibroblasts⁴⁰, and MOLT-4, a T-cell leukaemia-derived cell line which does not express the EGF receptor.

* PDL, population doubling level; PLC, per cent replicative lifespan completed.

unique protease, enhanced expression of a constitutively produced protease, or slower regeneration of receptor kinase activity might occur in senescent cells. In this light, it is perhaps relevant that increased plasminogen activator production accompanies receptor down-regulation in A431 cells³². It is also possible that receptors from senescent cells are more susceptible to artefactual proteolysis during sample preparation³³, although it would be difficult to correlate such an experimentally introduced event with the reduced mitogenic responsiveness of senescent WI-38 cells to EGF. Moreover, preincubation of extracts from young and senescent cells does not diminish the *in vitro* [γ -³²P] labelling of the EGF receptor in immune complexes (unpublished observation).

The inability of the EGF receptor from senescent WI-38 cells to be autophosphorylated at tyrosine is the second specific biochemical marker to be associated with *in vitro* ageing, the failure of senescent cells to initiate DNA synthesis being the first. It will be of interest to determine whether diminished autophosphorylating kinase activity is restricted to the EGF receptor or applies to other tyrosine protein kinases as part of a general phenomenon, and whether senescence of WI-38 cells is the result of diminished EGF receptor autophosphorylating kinase activity or its cause. Regardless, restoration of inherent tyrosine kinase activity may be one mechanism by which transformation is achieved in cells otherwise programmed to senesce.

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Phosphorylation-dependent contraction of actomyosin gels from amphibian eggs

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Actin networks or gels are a major structural component of the cytoplasm in most eukaryotic cells, especially those exhibiting motility^{1,2}. Cell-free extracts from a variety of cell types, including sea urchin eggs³ and *Xenopus* oocytes⁴, have been successfully used to examine the regulation of actin gelation and contraction. Although it has been shown that actin gel contraction is myosin-dependent (for review, see ref. 1), nothing is known of the role of myosin phosphorylation in this process. Indeed, much of the evidence suggesting that actomyosin-dependent movement in nonmuscle cells is regulated by myosin phosphorylation is provided by experiments showing that phosphorylation affects actin-activated ATPase activity^{5,6}. Here we report that the rate of actin gel contraction is increased, and several additional gels form and contract, in extracts from *Xenopus laevis* eggs prepared in conditions that promote protein phosphorylation. Since, in these conditions the 18,000-molecular weight (MW) light chain of myosin is the major phosphorylated component of the contracted gels, these results demonstrate a direct relationship between myosin phosphorylation and actin gel contraction. Evidence is also presented demonstrating that the recurrent waves of gelation and contraction occur in the absence of fluctuations in free-Ca²⁺ or pH.

Procedures used for preparation of high-speed cytoplasmic extracts (HSE) from *Xenopus* eggs are outlined in Fig. 1. When HSE is incubated at 18–25 °C, a distinct gel forms which slowly contracts over a period of 12–24 h. Actin is a main component of the gel as shown by the following criteria: (1) a major band co-migrates with *Xenopus* egg actin in SDS-polyacrylamide gel electrophoresis (SDS-PAGE, see Fig. 1)⁷, (2) there is a predominance of 6–7 nm filaments which bind myosin subfragment-1 when examined by electron microscopy, and (3) gelation is inhibited by cytochalasin B and not by colchicine. Gel contraction is myosin-dependent since it is inhibited by *N*-ethylmaleimide-modified heavy meromyosin (NEM-HMM), a specific inhibitor of actomyosin interactions⁸. Also, SDS-PAGE electrophoresis of contracted gels shows the presence of a protein which co-migrates with *Xenopus* egg myosin (Fig. 1). This protein is absent in uncontracted gels (see Fig. 1).

When 2 mM ATP (adenosine 5'-triphosphate) is added to HSE incubated at 22 °C, gel contraction occurs by 18 h. However, in the presence of 2 mM ATP γ S [adenosine-5'-O-(3-thiotriphosphate)], gel contraction occurs by 90 min. Further acceleration of gel contraction results when ATP γ S is added during preparation of HSE (see Fig. 1). Other studies have shown that when ATP γ S is used as a substrate by myosin light chain kinase in permeabilized smooth muscle cells, the 20,000-MW light chain of myosin is irreversibly thiophosphorylated, resulting in activation of myosin ATPase activity and development of tension⁹. In gels which have contracted in the presence of [³⁵S]ATP γ S, the 18,000-MW light chain of myosin is labelled (Fig. 1D). Labelling of the 15,000-MW light chain or 200,000-MW heavy chain of myosin is not detected. Although the 18,000-MW protein is the most prominent, other minor components of the contracted gel are also labelled (see Fig. 1D). Acceleration of gel contraction is also observed with HSE prepared in the presence of the phosphoprotein phosphatase inhibitors NaF and Na-glycerol-PO₄.

In conditions used to promote irreversible phosphorylation, several additional gels form and contract in the same aliquot of

incubated HSE. This is illustrated in Figs 2–4. These waves of gelation and contraction are actomyosin-based as shown by SDS-PAGE electrophoresis (see Fig. 1). Also, gelation is inhibited by cytochalasin B and contraction by NEM-HMM. Each wave of gelation and contraction appears to be autonomous since the contracted knot of each gel formed can be removed without disturbing the time course of gelation and contraction of the next wave. This result does not rule out the possibility of recycling occurring during the waves, since components (such as actin-binding proteins) could be released during gel contraction and incorporated into the next gel formed.

Calcium is an important regulator of actin gel formation and contraction¹. Using a Ca^{2+} -selective electrode¹⁰ and arsenazo III (a metallochromic indicator dye¹¹), we determined whether fluctuations in free- Ca^{2+} occur during the waves. Results show no fluctuation in free- Ca^{2+} during waves induced by either ATP γ S or phosphatase inhibitors. The free- Ca^{2+} of the HSE preparations was found to vary between 0.2 and 0.5 μM . Also, the waves of gelation and contraction proceed in the absence of a fluctuation in free- Ca^{2+} since, using Ca^{2+} /EGTA buffers¹²,

they continue to occur when the free- Ca^{2+} in the HSE is adjusted to defined levels between 0.01 and 10 μM . The greatest number of waves occur in 0.1–1 μM free- Ca^{2+} . Ca^{2+} is required for gelation and contraction since in the presence of >20 mM EGTA, the rate of gel contraction decreases and the total number of waves are reduced as the amount of EGTA is increased. Since a fluctuation in free- Ca^{2+} is not required for the waves to occur, it appears that under these conditions Ca^{2+} is a permissive co-factor, not a required trigger.

Actin gels from extracts of *Dictyostelium* have been induced to contract by raising the pH above 7.0¹³. Although the pH of the HSE preparations varied between 6.9 and 7.1, measured using microelectrodes¹⁴, no fluctuation in pH was detected in HSE undergoing multiple waves of gelation and contraction (I.D. and R.M.E., unpublished data).

To examine whether enhanced myosin light chain phosphorylation alone is responsible for accelerating gel contraction and promoting multiple waves, experiments were done whereby myosin light chain kinase (MLCK; purified from chicken gizzard¹⁵) is added to HSE before warming. In contrast to untreated HSE, which forms a gel that contracts by 18 h, gel contraction

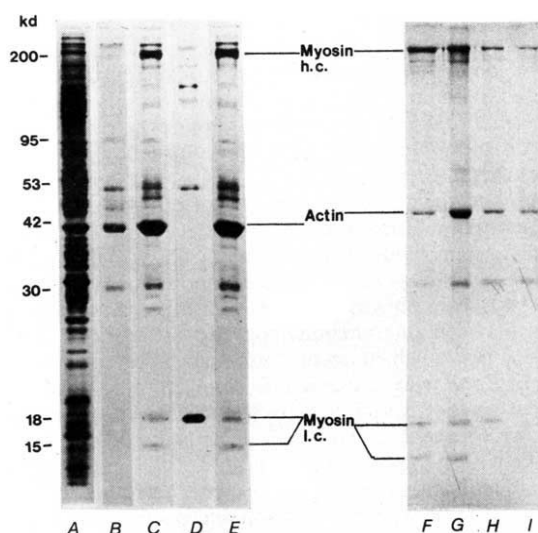


Fig. 1 0.1%-SDS-10% polyacrylamide slab gel electrophoresis⁷ of whole *Xenopus* HSE and uncontracted and contracted gels isolated from HSE incubated at 22 °C. Lane A, whole unwarmed HSE; B, uncontracted gel from HSE incubated with 2 mM ATP for 2 h; C, contracted gels (includes those resulting from multiple waves) from HSE incubated with 2 mM [³⁵S]ATP γ S (0.4 Ci mmol⁻¹ ATP γ S diluted from 59 Ci mmol⁻¹ [³⁵S]ATP γ S) for 2 h; D, fluorograph of lane C showing incorporation of ³⁵S into the 18,000-MW light chain of myosin; E, contracted gels from HSE prepared in the presence of phosphatase inhibitors incubated for 2 h (its composition is similar to the gels contracted in the presence of ATP γ S—see lane C); lane F, actomyosin isolated from whole *Xenopus* HSE using a procedure modified from Trotter and Adelstein²⁴. Lanes G–I are contracted gels from the first (G), second (H), and third (I) waves in HSE incubated at 22 °C which was prepared in the presence of phosphatase inhibitors. Equal proportions of the contracted gels were loaded onto lanes G–I to illustrate that each contains less protein than the wave before it.

Methods: HSE was prepared by washing mature ovulated de-jellied eggs (ovulation was hormonally-induced with human chorionic gonadotropin) in a medium containing 250 mM sucrose, 200 mM NaCl, 35 mM imidazole-Cl, 4 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 0.5 mM benzamidine-HCl, and 0.3 mM phenylmethylsulphonyl fluoride (PMSF), pH 6.5. When preparing HSE in the presence of phosphatase inhibitors, NaF is substituted for NaCl and Na-glycerol-PO₄ for imidazole-Cl in the wash medium. All procedures were done at 2–4 °C. Washed eggs were packed by centrifugation at 300g for 5 min, excess medium was removed, followed by centrifugation for 45 min at 12,000g. The middle viscous fraction (ATP γ S added here when indicated) was then removed and centrifuged twice at 150,000g for 3 h. The resultant clear supernatant is the HSE. Additional PMSF (0.3 mM) was added to HSE at this point in its preparation. Protein concentration of HSE varied between 25 and 30 mg ml⁻¹.

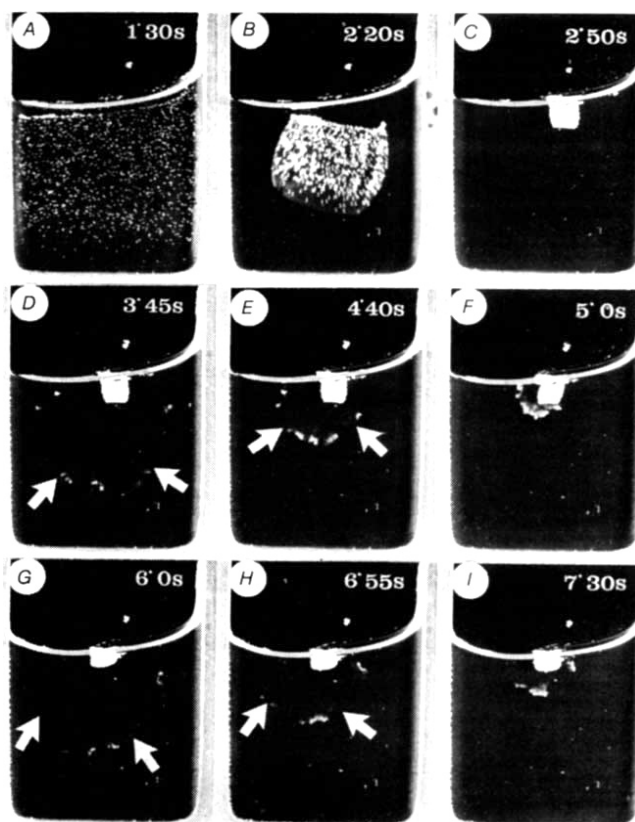


Fig. 2 A series of photographs illustrating three consecutive waves of gelation and contraction (A–C, D–F, G–I) occurring in a cuvette containing 0.2 ml of HSE (prepared in the presence of phosphatase inhibitors) incubated at 22 °C. Similar results are observed with ATP γ S added to HSE either during its preparation or before incubation. Times shown are in minutes and seconds and correspond to the time after start of incubation. The bubbles seen in the first contracting gel (A–C) are due to outgassing from warming the HSE. The contracted gels do not relax and as shown here, each new gel contracts towards the contracted knot of the previous wave. This is caused by attachment during gel assembly to the contracted knot. The gel formed during each wave occupies the entire volume of HSE in the cuvette. D and G were taken after gel contraction had begun and proceeded to where the outline of the contracting gel can be seen. As can be seen by comparing A, D, and G, each gel formed is less dense than the previous one. SDS-PAGE electrophoresis of contracted gels from the first three waves show that each contains less protein than the one before it (see lanes G–I of Fig. 1). Arrows in D, E, G, and H point to the edge of the contracting gel for the second and third wave.

occurs by 3 h and additional waves result in the presence of MLCK (see Fig. 4A). When unregulated MLCK is used, gel contraction is further accelerated and more waves occur (see Fig. 4B). It is possible that unregulated MLCK, which has its regulatory site removed by proteolysis¹⁶, is more effective because it is not dependent on elevated Ca^{2+} and calmodulin to phosphorylate myosin^{17,18}. Although the time course of waves induced by unregulated MLCK is similar to HSE incubated with ATP γ S (see Fig. 3C), gel contraction is not as extensive for HSE incubated with either regulated or unregulated MLCK. Since phosphatase inhibitors were not present during incubation of HSE with MLCK, the reduced gel contraction could be due to endogenous phosphatases decreasing the amount of myosin phosphorylated by MLCK. This possibility is supported by preliminary experiments whereby in HSE which had both ATP γ S and MLCK added before incubation, the rate of gel contraction and number of waves is increased tenfold, resembling that observed for HSE prepared in the presence of ATP γ S (see Fig. 3B).

We propose that the multiple waves of gelation and contraction, which occur under conditions of irreversible phosphorylation, are due to permanently turned on (that is, phosphorylated) myosin contracting gels before they can incorporate all of the available actin and other proteins involved in gelation. As a result, these remaining components are utilized for the next round of gelation and contraction. However, the conditions used in these experiments to enhance protein phosphorylation are

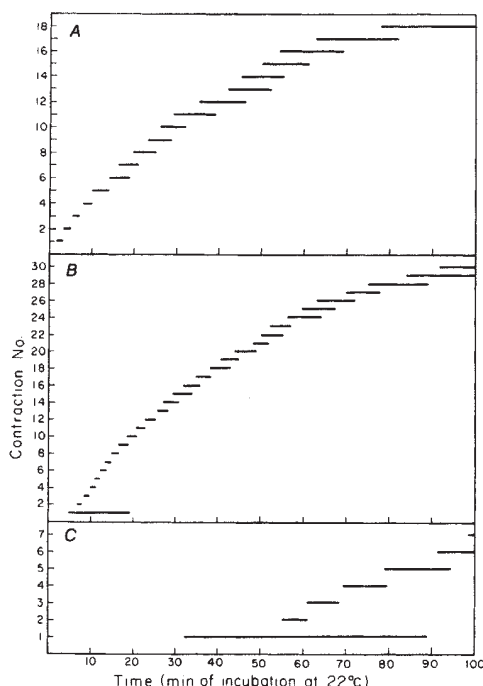


Fig. 3 Time course of waves of gelation and contraction. Data were obtained by analysing time-lapse video tapes of HSE incubated in a cuvette at 22°C. Each line represents the duration of contraction for each gel formed. **A**, HSE prepared in the presence of phosphatase inhibitors; **B**, HSE prepared in the presence of 2 mM ATP γ S; **C**, 2 mM ATP γ S added to HSE before incubation. See Fig. 2 for corresponding visual representation of **A**. The time course is temperature-dependent since the rate of gel formation and contraction increases as the temperature is raised. Note that the duration of contraction increases in comparison to the previous wave. In **A**, by the seventh round of gelation and contraction, a newly formed gel begins to contract while the previous one is still contracting. This overlap in gel formation and contraction begins earlier in **B** and **C**. Gel contraction is faster and more waves occur when ATP γ S is added during HSE preparation (**B**) than when it is added to HSE before incubation (**C**). These differences may be due to more thiophosphorylation occurring when ATP γ S is present during HSE preparation than when it is added to HSE before incubation.

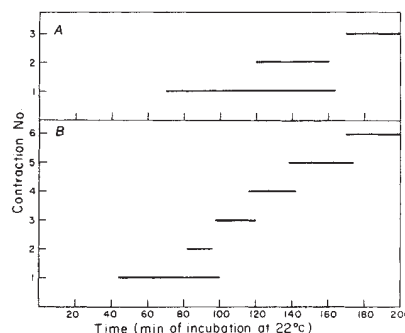


Fig. 4 Time course of waves of gelation and contraction in HSE incubated with **A**, intact regulated MLCK, and **B** unregulated MLCK. See Fig. 3 for explanation of graphs. HSE contained 65 $\mu\text{g ml}^{-1}$ MLCK for both **A** and **B**. Unregulated MLCK was prepared from intact MLCK by tryptic digestion¹⁶. Addition of regulated MLCK accelerates gel contraction and induces multiple waves. Unregulated MLCK is more effective.

not restricted to only increasing myosin light chain phosphorylation. Recent studies have reported phosphorylation of actin-binding proteins such as filamin and spectrin^{19,20}; and as shown in Fig. 1D, other components of the contracted gel are thiophosphorylated. Although we have no evidence for this, it is possible that phosphorylation of other proteins besides myosin light chain may play a role in accelerating actin gel contraction and inducing multiple waves.

To our knowledge, no one has reported observing several gels sequentially form and contract in cell-free cytoplasmic extracts. It seems likely that these recurrent waves of gelation and contraction are a consequence of conditions which increase myosin light chain phosphorylation, and it is tempting to draw parallels between this phenomenon and the periodic cortical contraction waves which occur during early *Xenopus* development²¹. In *Rana pipiens*, cleavage is believed to be actomyosin-dependent since it is inhibited by NEM-HMM²². Also, as has been shown for the *Ascidian* egg²³, an actin-based cytoskeleton may participate in the cytoplasmic localization of morphogenetic determinants in the *Xenopus* egg. Further examination of the regulation of these waves of gelation and contraction may give new insight into understanding actomyosin-based movements in nonmuscle cells and during development.

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Frank Yang and physics

P.T. Matthews

Selected Papers 1945–1980 With Commentary. By Chen Ning Yang.
W. H. Freeman: 1983. Pp.596. Hbk \$43.95, £41.50; pbk \$21.95, £18.95.

WHEN distinguished physicists reach the age of sixty — or some similar milestone — it is not unusual to celebrate the occasion by bringing out a festschrift made up of papers written on request by friends and colleagues. These volumes must be a source of satisfaction to the person so honoured but are seldom of much significance in the scientific literature. Good papers are not often written to order in this way.

Professor Yang — Frank Yang as he is known in the West, Yang Chen Ning to his fellow Chinese physicists with whom he has re-established contact — has wisely chosen a different course. This book celebrates his sixtieth birthday by reprinting a selection of his papers with his own commentary. The commentary is partly autobiographical but also serves to set his own work within the context of the advances being made in the relevant fields at the time and to show the interplay between the development of his own thinking and this general background. The commentary is totally unpretentious, straightforward and unassuming. Some seventy papers are reproduced out of a total of over two hundred published by Yang between 1945 and 1980.

Yang's two main fields of interest have been in statistical mechanics and in symmetry principles in relation to elementary particles. He has remained active at the frontiers in both these areas and this collection of papers also provides, from his own viewpoint, a history of the main developments in these fields.

In the years immediately following the Second World War very rapid advances were made in elementary particle physics, exploiting the theoretical ideas of Yukawa and Kemmer and the flood of new experimental information which became available with the increasing energy of particle accelerators. Simultaneously, theoretical physicists were fascinated by the remarkable properties of superconductors and superfluids and the question of whether these phenomena could be understood in terms of quantum statistical mechanics or if they pointed to new fundamental effects outside the conventional framework. Under the early influence of Fermi, Yang has maintained an active interest in both these areas. He has contributed over the years to the general problem of phase transitions and the insights which can be gained from simplified systems more amenable to mathematical analysis, such as the Ising model, the one-dimensional problem and the hard sphere gas. But the main thrust of his collected work is in elementary particle theory.

Historically the most fascinating aspect of this record of achievement is the account of the establishment of parity violation in weak interactions and the subsequent recognition of the fundamental role of the



Heads together — C.N. Yang (left) and T. D. Lee at the Nobel prize ceremony, December 1957.

CTP theorem during 1956 and 1957. For the part which he played in this Yang was awarded the Nobel Prize. His other major contribution, which took much longer to mature, but in the end has had possibly an even greater influence on the development of the subject is his work completed in 1954 in collaboration with R.L. Mills, on isotopic spin conservation and generalized gauge invariance. This is the first example of a quantum field theory which is gauge invariant with respect to a non-Abelian group. The subsequent developments, particularly those of renormalization by G. 't Hooft and the electroweak theory of Glashow, Salam and Weinberg, have made

this the central basic concept in our understanding of all the fundamental forces in nature, according to current thinking. The Yang–Mills paper started the chain of ideas which led to the recent discovery at CERN of the W and Z intermediate bosons.

These two outstanding pieces of Yang's work are not isolated achievements, but stand out as high points against a background of significant contributions to physics over a period of nearly forty years. The published papers speak for themselves, but combined with Yang's own commentary in this book, the net effect is a fascinating insight into the workings of a first rate scientific mind. It also gives a vivid impression of a very able, very sincere and a very likable person who has obviously derived immense satisfaction from his work. This impression is enhanced by a number of photographs in many of which Yang himself appears with a succession of other physicists with whom he has collaborated over the years. One is reminded of W.B. Yeats' phrase describing two anonymous Chinese from an earlier epoch. His "ancient glittering eyes are gay". They have good reason to be.

I can recommend this book strongly to anyone seriously concerned with the development of fundamental physics since the Second World War. With the help of the commentary, it should also be of interest to a wider public, who can get a very clear impression of what it is like to devote one's life successfully to what at one time was somewhat denigratingly classified as "curiosity based" research, warts and all, including the strains on personal relationships which can easily follow from public recognition of collaborative work. □

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Low profiles

Peter Liss

Trace Metals in Sea Water.

Edited by C.S. Wong, E. Boyle,
 K.W. Bruland, J.D. Burton and
 E.D. Goldberg.

Plenum: 1983. Pp.920. \$115, £80.50.

IN his Presidential Address to the Geochemical Society in 1976, Karl Turekian said "... one has the feeling that the whole field of trace metal marine geochemistry would have been a completely dull one over the past fifty years if it weren't for analytical errors! The startling conclusion, as the sources of errors become better understood and the quality of the analyses improve, is that most trace metals are at extremely low concentrations in the oceans and have

rather unspectacular variations in their concentrations".

Measurements made since then have added weight to Turekian's judgement which, at the time of his address, was made on the basis of very few reliable results. We now know that much of the earlier trace metal data for seawater was plagued with errors due to sample contamination during collection, storage and laboratory processing. With the adoption of "clean" sampling and analytical techniques, pioneered by Clair Patterson for lead, reliable data are now appearing. Consequently, although reports of abrupt and spectacular changes in seawater trace metal concentrations have declined, trace metal distributions are not homogeneous and hence are still of great interest. It is becoming clear that trace metal distributions are closely related to other oceanographic phenomena.

For example, the metals cadmium, zinc

and nickel show distributions strikingly similar to those of the well-studied nutrient elements, especially phosphorus. These metals are found at their lowest concentrations in near-surface waters where they are taken up by marine organisms. Deeper in the water column, as the sinking organisms decompose, the metals are regenerated, return to the aqueous phase and result in enhanced concentrations at depth. This similarity in behaviour, and hence vertical concentration profile, leads to the idea of these metals and plant nutrients exhibiting Redfield-type ratios in the oceans. The extent to which there exists such a constancy of metal: nutrient ratios, and for which elements, remains to be firmly established as more rigorously obtained results become available. Even now there is evidence that deep water concentrations of several trace metals are higher in the Pacific Ocean than in the Atlantic, a situation which again mimics the behaviour of the conventional nutrient elements.

However, not all trace metals show nutrient-type behaviour. For example, manganese and lead are rapidly removed from solution in much of the water column. But for manganese there is sometimes also addition to the water by conversion of insoluble Mn (IV) to soluble Mn (II). Recent laboratory studies indicate that, near the sea surface, this change may be photochemically mediated and could explain the near-surface concentration maxima for dissolved manganese observed in some profiles. Furthermore, the oceanic distribution of both metals is strongly influenced by riverine and atmospheric inputs, which for lead have increased substantially in recent years because of human use.

The present book is essentially the proceedings of a meeting in Sicily in the spring of 1981, at which these issues were considered. It contains 47 papers, which at the conference were divided into seven sections. As at the conference two topics — oceanic distribution and analysis, and chemical speciation (eleven papers each) — are especially well covered. This contrasts with the section on air/sea exchange plus coastal processes which contains just four papers. All the chapters have been reviewed and this has led to a two-year delay in publication. In a fast-moving field like this such a hiatus was unfortunate and inevitably lessens the book's impact somewhat.

However, such criticisms are relatively minor when viewed against the very substantial achievement of the book's editorial board in bringing together in one volume such a large proportion of our recent knowledge in this area. Every laboratory where trace metals in seawater are studied should have easy access to it. □

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Biology: the search for culture

Robert Attenborough

Human Culture: A Moment in Evolution.

By Theodosius Dobzhansky and Ernest Boesiger. Edited and completed by Bruce Wallace.

Columbia University Press: 1983.

Pp.175. \$24.50.

Ever-Expanding Horizons:

The Dual Informational Sources of Human Evolution.

By Carl P. Swanson.

University of Massachusetts Press: 1983.

Pp.162. Hbk \$13.50, pbk \$7.50.

EVOLUTIONARY theory is as attractive to biologists now as it has ever been. Most social scientists, however, continue to resist its allure, recalling the malign influence of nineteenth century evolutionism in the social arena. It is no surprise, then, that these two books on the relationship between biological and cultural evolution should both approach the problem from the biological end.

Both books are written by senior figures in biology, distinguished in their own fields, and both are very sane and literate treatments, pitched at a serious but non-technical level and pleasingly well produced. But in the end, sadly, both books are unsatisfying.

Human Culture is based on lectures which Dobzhansky gave at the Collège de France in 1974. With Boesiger, he planned a book on the basis of these lectures, but both men died in 1975, leaving their manuscript incomplete. Wallace was then asked to edit and complete it — a task which was apparently made harder by his own temperamental disinclination to write in the grand manner about "the majestic sweep of human evolution".

Despite its title, the book as it now emerges begins with a survey of general evolutionary topics that has little specific bearing on human evolution, let alone human culture. A particular concern here is to expose vitalism, preformationism, and other such -isms, especially in their modern manifestations (for example Grassé, author of *Evolution of Living Organisms*, is criticized for vitalistic teleology). The overall argument amounts to an orthodox defence of modern synthetic evolutionary theory, with a balanced appraisal of the "non-Darwinian" neutralist hypothesis, and stressing the interactions between genes and environment and the prevalence of polymorphic variation.

When the authors do turn their attention to the human species, they proceed by a series of brief discussions, on the evolution of the human body and brain, and on the evolutionary emergence of human culture, including language, self-awareness, death-

awareness, ethics and (more discursively) aesthetics. They also compare biological and cultural evolution, and present a historical perspective on evolution and human origins, from the Vedas and Taoism to our century, with particular attention to Buffon, Lamarck and Cuvier ("a spiteful dictator in biology"). Finally there is a discussion of human equality and diversity, concentrating on the IQ controversy.

In all this, the sheer breadth of scope brings dangers, which are not successfully avoided. The most damaging criticism of the book is not, in my view, that it is wrong (though I do have some disagreements). Rather it is that, through an excess of bland, diffuse generalizations, it fails to say much on any particular issue that is really distinct or constructive. This might have been remedied, had the authors lived to complete their book, but it would have required a more root-and-branch revision than could be expected of an editor. A further weakness is the simple fact that the book was written nearly a decade ago, and now seems dated. What Dobzhansky and Boesiger might have made of recent developments in molecular genetics, neo-Lamarckism, macroevolution, sociobiology, the IQ debate and theories of culture, we can only guess.

Like Dobzhansky and Boesiger, Swanson in *Ever-Expanding Horizons* portrays cultural evolution as both emergent from and analogous to biological evolution, though he is more explicit about pursuing the analogy. Also like them, he gives a considerable portion of his book over to general topics in evolution and genetics — more than seems relevant to his argument — but he also discusses, a little more substantially, the evolution of the human body and brain, and the evolutionary origins of human culture, language and so forth. His main approach when it comes to discussing cultural evolution as such is to propose the "sociogene" (meaning, roughly, concept) as the cultural counterpart of the "biogene", and to extend the principles of evolutionary genetics to cultural processes.

Although not quite so ambitious as *Human Culture*, this too is a very wide-ranging book, and it suffers from a similar principal failing. Much of what Swanson says sounds unexceptionable, even impressive, but often it is too abstract to advance the argument very far. Beyond the generalities, there is a great dearth of relevant detail, especially on the cultural side. If the author does, as the dustjacket boasts, have an "extensive knowledge of . . . cultural anthropology" as well as biology, that is not apparent in the course of the book.

As to "sociogenes", ideas of this kind have been proposed before (e.g. Dawkins's "memes"), and indeed Swanson reviews some of them. So something more substantial than just playing with the idea might be expected from a new contribution — such as genuinely fresh insights or

testable predictions. But when, for example (in one of his few brief references to any actual culture), Swanson describes Protestantism as "an allele of Christianity", all he appears to be doing is re-phrasing common knowledge in genetic language. This seems to typify his approach. Little justification is offered for seeking the cultural analogues of genetic processes, from replication and recombination to drift and selection, or for supposing that the identification of such analogues in itself enhances understanding.

Both books, then, attempt much and achieve disappointingly little. Neither is without some redeeming qualities, and no doubt each will have an interested audience. But I could not recommend either of them to biologists or general readers in search of an incisive, informative view of human biology and culture: nor could I assure cultural anthropologists that either would offer them convincing grounds for taking evolutionist views of culture more seriously. □

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Scattered thoughts

Z. Kam

Absorption and Scattering of Light by Small Particles.

By Craig F. Bohren and Donald R. Huffman.

Wiley: 1983. Pp. 520. £42.70, \$59.80.

BLUE skies and red sunsets intrigued Leonardo de Vinci and stimulated some of the best scientific compositions of Tyndall and Lord Rayleigh. While no longer an enigma to a generation which was exposed to essays on sky colour in the classics of children's scientific literature and which witnessed colour television images of lunar sunrise, the interaction of radiation with inhomogeneous media is nevertheless a subject of research where applications are so varied that it is difficult to integrate, into

End of Bewley & Black

SPRINGER-Verlag have recently published both the long-awaited second volume of J. Derek Bewley and Michael Black's *Physiology and Biochemistry of Seeds in Relation to Germination*, and a corrected reprint of Vol. 1. The first volume, which appeared in 1978, deals largely with the germinating seed, while Vol. 2 is devoted to aspects of dormancy. Prices are Vol. 1 DM 97, \$41, and Vol. 2 DM 128, \$55.

Paper phenotype

RICHARD Dawkins's *The Extended Phenotype* has now appeared in paperback (for review see *Nature* 296, 506; 1982). Price is £5.95, \$7.95; the publisher is Oxford University Press.

one essay, the theories and methodologies used in different fields.

One therefore inevitably assumes a somewhat subjective point of view in approaching Bohren and Huffman's *Absorption and Scattering of Light by Small Particles*. In fact, the book is quite well written and contains the basic theory. The relation of scattering and absorption phenomena to the bulk properties of the scatterers is logically developed through rigorous derivations; examples are easy to follow and adequately illustrate the theory. The chapters contain more than enough cross references to create a balanced, well integrated presentation.

The basic theory of scattering and absorption by particles is preceded by a concise summary of electromagnetic wave phenomena in the first chapter. The next chapter presents the classical approximations for particles smaller than the wavelength of the radiation, the Rayleigh-Gans approximations, Mie scattering and a few other exact solutions. The optical properties of bulk matter are then related to absorption and scattering of suspensions of particles. The chapter about applications is limited chiefly to examples from atmospheric and space sciences.

Although the authors restrict themselves to small-particle light scattering in this volume, I think that some additional reference to scattering and absorption of ultraviolet and X-ray radiation, as well as of neutrons and electrons, would have been appropriate and stimulating, in view of the extensive development of such modern techniques as synchrotron radiation spectroscopy and electron microscopy. The same can be said for limiting cases of scattering from dense suspensions of particles and plasmas.

It is unfortunate — at a time when students' imaginations are more likely to be stirred by problem-oriented approaches to basic scattering theory than by a formal exposition, however well-presented — that the authors give modern, practical applications considerably less attention than seems warranted. While the basic theory is developed on occasion to a level of computer algorithms, the presentation of other applications, in short descriptive passages, often lacks depth. It is consequently easy to relate basic theories to practical problems in meteorology, biophysics or fibre optics. Without providing motivation for developing appropriate models for the understanding of complex phenomena, the task of applying the theory presented in the book to new problems may well be left to the very few physicists who will choose this area as their field of research. In this sense, the book lacks the potential to become a classic reference but is, nonetheless, an important and well-written update to the established references such as those of Kerker and van de Hulst. □

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The rhythm of Barro Colorado

Malcolm Coe

The Ecology of a Tropical Forest: Seasonal Rhythms and Long Term Changes.

Edited by E.G. Leigh, A.S. Rand and D.M. Windsor.

Oxford University Press/Smithsonian Institution Press: 1983. Pp. 468. Pbk £19, \$15.

IN 1980, T.C. Whitmore wrote: "The second half of the twentieth century will stand in history as the brief period during which Man reduced the area of the richest and most complex ecosystems in the world by one third". The ecosystems he referred to, of course, are the world's tropical rain forests where evolution seems to have gone mad, producing a vast and, in the large part, unstudied flora and fauna. These forests straddle the Equator in West Africa, South East Asia and South America, and together comprise just over 50% of the Earth's forests. The largest of these forests are in South America, where in 1976 A. Sommer calculated that no more than 63% of the forest area still survived.

It is against a background of ruthless exploitation and destruction that we must view this collection of papers which was first published by the Smithsonian Institution in 1982. The work presented was entirely carried out on Barro Colorado Island by staff, students and visitors to the Smithsonian Tropical Research Institute in Panama. This island is a basaltic hill, which was previously submerged when the Gatun Lake, in which it lies, was formed in 1914, and now covers an area of 1500 ha.

Ecological and behavioural studies are often carried out in a specific area because a particularly interesting species happens to be present, which in consequence results in a poor knowledge of local physical conditions and their accompanying animal and plant communities. Indeed, there are probably no more than a few dozen areas in the world that have been submitted to detailed inventory studies accompanied by long-term monitoring programmes of the local biota. The detailed background to many of the papers in this volume derives from such an environmental monitoring programme, established on Barro Colorado Island as long ago as 1916. Although the small size of the island may have led to artificially high densities of some animals, the seasonal nature of this isolated, tropical deciduous forest provides a valuable contrast with studies carried out in similar environments on other continents, and even with the richer humid tropical forests.

The value of this volume to tropical ecologists is that it draws together information on the physical factors which

determine the seasonal rhythms, which in turn characterize the plant species making up the major structural elements of the major forest habitats. Against this background the reader is led through the manner in which arboreal and terrestrial frugivores utilize the forest's primary production, and the role of insects and arthropods which feed in the canopy and on ground litter. The final section will be of special interest to those who study similar conservation areas that are defined by distinct physical boundaries limiting the dispersal of elements of the flora and fauna. One of the editors, Egbert Leigh, provides a theoretical background to the two important studies of temporal variations in populations of birds and terrestrial mammal communities, with the latter revealing 29,000 of the larger components on 1500 ha, a high density of

over 19 animals per hectare.

This volume will stand alongside H. T. Odum and R. F. Pigeon's *A Tropical Rain Forest* (US Atomic Energy Commission, 1970) and A. R. E. Sinclair and M. Norton-Griffiths's *Serengeti — the Dynamics of an Ecosystem* (Chicago University Press, 1979) as vital reading for biologists who wish to view tropical ecosystems as a whole. It may also encourage those funding tropical ecological research to initiate similar broad-based studies in other parts of the world, for the fashionable demand for environmental impact statements and management plans has little meaning without the collection of such basic data. □

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Anomalous science

V.E. Viola

The Origin of the Chemical Elements and the Oklo Phenomenon.

By P.K. Kuroda.

Springer-Verlag: 1983. Pp.164. DM 92, \$39.70.

FASCINATING insights into the evolution of nature's elements and the subsequent history of our Solar System can be obtained from the study of elemental abundances and isotopic anomalies in terrestrial, meteoritic and lunar materials. In this book, Paul Kuroda examines some of the facets of this subject with which he has been involved in his distinguished research career.

The book has a distinct historical flavour, containing an extensive list of early references, but it also highlights several significant areas of current research in geo- and cosmochemistry. The title is somewhat misleading, however, as the emphasis is not so much on the origin of the elements as it is on the search for rare nuclear species in nature and the perturbations of the major nucleosynthetic processes that have produced observed anomalies in normal abundances.

The author introduces his subject by tracing the efforts to compile a uniform table of Solar System abundances. The account falls somewhat short, however, in that it does not extend to the inclusion of currently-used tables, nor does it examine the complexities inherent in generating such compilations. However, in Chapter Three attempts to detect ^{43}Tc and ^{61}Pm , absent in nature due to their radioactivity, are discussed and this emphasizes the critical role of analytical sensitivity in establishing the existence of rare or anomalous isotopic species in natural materials.

The most significant contributions are

found in Chapters Four and Six. The former deals with the Oklo phenomenon — the extinct natural nuclear reactor discovered in the Republic of Gabon, in 1972. Beginning with the factors which led the author to predict the existence of such a reactor in 1956, both the theory of natural reactors and the experimental efforts to detect them are extensively discussed. Chapter Six focuses on the extinct radio-nuclides ^{244}Pu ($t_{1/2} = 8.1 \times 10^7 \text{y}$) and ^{129}I ($t_{1/2} = 1.6 \times 10^7 \text{y}$). In addition to describing attempts to detect primordial remnants of these radioactive species, the author stresses their important relationship to the interpretation of xenon isotope ratios in natural materials.

Theories of nucleosynthesis are dealt with in Chapter Five, the coverage of this topic being largely historical in nature, while in the final chapter the author proposes a unified theory to account for the unusual isotopic abundances found in certain meteorites (R.N. Clayton, *et al.*, *Science* **182**, 485; 1973). He begins by examining the origin of the rare elements Li, Be and B and the meteoritic isotope anomaly data. (A major Li Be B reference is omitted from these discussions, S.M. Austin, *Prog. Part. Nucl. Phys.* **40**, 14; 1980). Then it is postulated that the nuclear processes responsible for Li, Be and B synthesis were instrumental in the creation of the isotopic anomalies observed in meteorites. However, in relating these two phenomena, the author assumes that all ^7Li in the Solar System is produced by cosmic ray interactions. Because only about 10% of Solar System ^7Li is presently believed to be of cosmic ray origin, this analysis is not compelling.

However, the chapter concludes with a summary of isotope anomalies in several elements, which serves as a useful indication of the complexity and future promise for intriguing results in this field. □

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Wild orchids

M.A. Clements

Wild Orchids of Britain and Europe.

By P. and J. Davies, and A. Huxley.

Chatto & Windus/The Hogarth Press: 1983. Pp.304. £9.95.

At first sight this book appears to be all that English-speaking orchid enthusiasts have been waiting for — an authoritative up-to-date account of European orchids. Indeed, it is claimed to be so by the authors. The photographs are excellent but, unfortunately, the text does them scant justice as it contains scientific inaccuracies, half truths and generalizations many of which are untrue. There are, in fact, errors on nearly every page of the book, some minor but others of a more serious nature. For example, in the introductory chapter there is a section on seed germination in which most of the information is out of date by some 30 years or more. The authors have also perpetuated errors that have existed in the literature for decades. For example available evidence from *in vitro* germination of orchid seeds shows that it takes but a few months and not years, as is suggested on pages 6 and 8, for the first leaf of most orchids to emerge from the protocorm.

Several comments made in the text about particular name changes of orchids that the authors dislike illustrate their lack of understanding of the taxonomic method. For example, in the section on classification the reader might gain the impression that most botanists are incapable of correctly classifying and naming European orchids; in fact, the naming of plants, including orchids, is strictly controlled by the International Code of Botanical Nomenclature. Clearly the authors have little, if any, understanding of the rules governing the naming of plants. Thus, *Ophrys holoserica* is the only correct scientific name for the Late Spider Orchid. The authors prefer to use a name reduced to synonymy several years ago. Unfortunately, this is not an isolated example.

Scientific terms have been frequently misused in the text. In botany, it is more usual to speak of populations of plants rather than "races", a term more appropriate to zoology. Terms such as classes, tribes, genera, forms etc. are consistently misused, for example on page 8 where *Ophrys* and *Orchis* are referred to as tribes instead of genera.

By all means buy this book — its illustrations alone warrant it. But beware of accepting the often inaccurate opinions that accompany them. □

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